Cofactor Requirements and General Characteristics of a Soluble Fatty Acid Elongating System from Mitochondria*

L. A. Mooney and E. J. Barron

ABSTRACT: A mitochrondrial fatty acid chain elongation system was extracted from rat liver mitochondrial acetone powder, and the reactants and cofactors involved were studied.

The coenzyme A (CoA) esters of fatty acids from 4 to 20 carbons could be elongated and acetyl-CoA was the 2-carbon donor for the elongation process. Malonyl-CoA was incorporated only after decarboxylation to acetyl-CoA. Maximum activity was dependent on both reduced diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide.

Work from Mead's laboratory (Steinberg *et al.*, 1956; Fulco and Mead, 1959) has shown that, *in vivo*, fatty acids (at least of the unsaturated type) could be elongated. Later, the same process was shown to occur *in vitro*, with saturated fatty acids (Harlan and Wakil, 1962, 1963). This reaction was catalyzed by liver mitochondrial and microsomal fractions, as well as a soluble system from mitochondrial acetone powder. The characteristics of this system were worked out using the latter as the source of enzyme and were found to be quite different from the *de novo* synthesis. Acetyl-CoA was utilized as the elongating unit and DPNH plus TPNH were the cofactors. ATP was required if fatty acyl-CoA was provided as substrate. Although acetyl-CoA appeared to be the elongating unit, Malonyl-CoA could also be incorporated into fatty acids. In the latter case, incorporation was found to be mainly into palmitic and stearic acids and was not restricted to the carboxy terminus, thus indicating separate pathways for the introduction of acetyl- and malonyl-CoA esters into fatty acids.

The results obtained by Nugteren (1965) could be considered to be in conflict with those of the above authors if it were not for the fact that his enzyme system was a microsomal preparation. Malonyl-CoA was found to be the elongating unit and TPNH the reductive agent. In addition, 3-keto-, 3-hydroxy-, and Δ^2-unsaturated fatty acids were isolated as intermediates of this process. This report describes an elongating system prepared from mitochondrial acetone powder and reaffirms the results obtained by Harlan and Wakil (1963).

Materials and Methods

All radioactive materials were purchased from New England Nuclear. Nonradioactive fatty acids were obtained from Calbiochem or Applied Science Laboratories, Inc. The 14:0[1] and 16:0 anhydrides were products of K & K Laboratories. Pyridine nucleotides, ATP, and CoA were from P-L Biochemicals, Inc.; Nutritional Biochemical Corp. supplied the avidin and biotin, both of which were used without further purification. Darco K-B charcoal was a product of Atlas Chemical Co. Radioactivity on paper chromatograms was detected with a Vanguard autoscanner 880. Centrifugations were carried out in a Sorvall RC-2 or SS-3 held at 0–4° and radioactive materials were assayed in a Tri-Carb Model 314 EX-1 liquid scintillation spectrometer.

Acetone Powder Preparation. Male, Sprague–Dawley rats, weighing approximately 175 g, were maintained on standard laboratory chow for at least a week before use. They were anesthesized with ether, their jugular veins cut, and the animals exsanguinated. The livers were removed and washed four times in cold 0.25 M sucrose. The liver mitochondria were isolated by the procedure of Hogeboom *et al.* (1948), using 8 ml of 0.25 M sucrose/g of tissue. The isolated mitochondria were resuspended by use of a Potter–Elvehjem homogenizer in 0.5 of the original volume of 0.25 M sucrose and resedimented. The washed mitochondria were then homogenized in a volume of 0.04 M potassium phosphate buffer (pH 7.1), equal to 0.2 of the original volume of sucrose solution used. This suspension was run slowly into 150 volumes of cold acetone, with brisk stirring. The solids were allowed to settle and the acetone decanted. The same volume of acetone was used to resuspend the material and the settling and decanting were repeated. The material was then slurried into a Büchner funnel fitted with Whatman No. 1 paper and filtered with suction, followed by washing successively with about 300 ml of cold acetone and three washes of 300 ml each of cold ether. Care was used not to allow the cake to dry between washes. The filter cake was spread onto a filter paper and allowed to partially dry in the cold room (4°) for 15 min. The material was placed in a

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[1] The shorthand designation of Ahrens *et al.* (1959) is used for identifying fatty acids. The numeral before the colon designates the number of carbons and that after the colon, the number of double bonds, while a superscript to the latter numeral designates the position of the double bond, numbering from the carboxyl group, *i.e.*, 14:0 and 16:0 are myristic and palmitic acids, respectively, while $18:1^9$ is oleic acid.

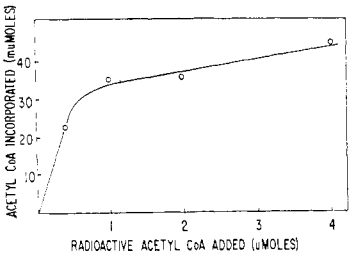

FIGURE 1: The effect of varying amounts of [1-^{14}C]acetyl-CoA on the amount incorporated into fatty acids. The assay mixture included 20 μmoles of ATP, 0.12 μmole of CoA, 0.4 μmole of DPN$^+$, 0.6 μmole of 16:1 potassium salt, 10 μmoles of Mn^{2+}, 100 μmoles of potassium phosphate buffer (pH 7.1), 15 μmoles of isocitrate, 5 μmoles of glutathione, and 1 μmole of acetyl-[1-^{14}C]CoA (sp act. 2 mCi/mmole). Final volume was 1 ml, and the time of incubation was 1 hr; 5.6 mg of protein was used in each sample.

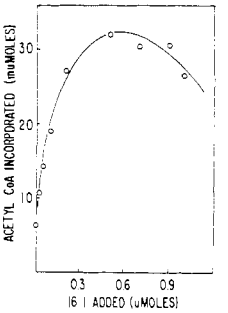

FIGURE 2: The incorporation of [1-^{14}C]acetyl-CoA into fatty acids in the presence of varying amounts of added 16:1 potassium salt. The conditions used were identical with those given in Figure 1.

vacuum desiccator and further dried at room temperature for 2 hr under a vacuum of 20 mm. The resulting powder was found to be stable for at least a year when stored at $-20°$.

Enzyme Preparation. The acetone powder was homogenized with a stirring rod in 0.4 M potassium phosphate buffer (pH 7.1), using a ratio of 70 mg of powder/ml of buffer. After the suspension sat at 0° for 20 min, it was centrifuged at 27,000*g* for 30 min. The supernatant was decanted, yielding approximately 28 mg of protein/ml (as determined with the biuret reaction of Gornel *et al.*, 1949). Charcoal-treated enzyme was prepared by suspending 20 mg of Darco K-B in each ml of supernatant. The mixture sat at 0° for 5 min, then centrifuged at 27,000*g* for 20 min. The clear, amber supernatant was passed, with the aid of slight pressure from a pipet bulb, through a medium sintered disk to assure complete removal of the charcoal. Only that portion of the solution which passed through the filter without foaming was used for the enzyme assay.

Fatty acid esterification was done with either diazomethane, using the method of Schlenk and Gellerman (1960), or BF_3–methanol as described by Metcalf and Schmitz (1961).

Hydrogenation of unsaturated fatty acids was carried out by the procedure of Vandenheuvel (1956) with 2 ml of methanol as solvent, 5 mg of the Pt–silicic acid catalyst, constant shaking, and a reaction time of 1 hr.

Synthesis of CoA Derivatives. Acetyl-CoA was synthesized by the method of Simon and Shemin (1953) and malonyl-CoA by the procedure of Trams and Brady (1960). Long-chain fatty acyl-CoA was obtained by the procedure of Vignais and Zabin (1958). In this latter procedure, the precipitated CoA derivatives must be washed with an acid medium, otherwise low product yields are obtained when using fatty acids shorter than palmitate.

Assay for Acetyl-CoA and Malonyl-CoA. The assay of Hatch and Stumpf (1961) was utilized in its essentials. A somewhat more detailed procedure with our modifications follows.

An aliquot of the enzyme reaction mixture (0.5 ml) was added to 1 ml of hydroxylamine solution (8% ethanolic NH_2OH–HCl neutralized with 4% ethanolic NaOH) followed by the addition of 0.5 ml of distilled water. The volume was reduced to 0.2 to 0.3 ml by heating to 40° under a stream of nitrogen. After acidification, the hydroxamates (and acids) were solubilized in 8 ml of acetone and the precipitated salts removed by centrifugation. The extract was then made alkaline with NH_4OH and the volume reduced. Aliquots were taken for paper chromatography (solvent systems used were: butanol saturated with 5 M formic acid and ethanol–ammonium hydroxide–water, 80:4:16; Aronoff, 1961).

Decarboxylation. The decarboxylation procedure of Schmidt as modified by Brady *et al.* (1960) was used to determine the amount of ^{14}C in the carboxyl carbon of fatty acids. Recovery and precision in our laboratory was determined by analyzing 31 samples of [1-^{14}C]palmitic and -stearic acids. After correcting for quenching by Hyamine, recoveries were found to be 91 $\pm$ 4%. Ten samples of uniformly labeled palmitic acid gave 104 $\pm$ 4.4% of the expected radioactivity ($^1/_{16}$) in the carboxyl carbon. It is likely that the uniformly labeled palmitic acid had a disproportionate amount of ^{14}C in the carboxyl carbon.

Enzyme Assay. The composition of the assay mixture is given for particular cases in the tables and figures. Incubation was always at 37°. After the appropriate time interval, the reaction was stopped by addition of 2 ml of 2 N methanolic KOH. The samples were saponified for 45 min at 70°, acidified with 2 ml of 3 N HCl, and extracted using the method of Bligh and Dyer (1959). Following these steps the samples were radioassayed.

Results

Optimizing Acetyl-CoA Incorporation. Incorporation of acetyl-CoA into fatty acids was shown to increase linearly with concentration of mitochondrial protein up to 6 mg/ml of assay mixture. All assays were therefore adjusted to contain 4–6 mg of protein. With this amount of protein, concentrations of the substrates, acetyl-CoA and fatty acid, were then made to be nonlimiting. Figures 1 and 2 show the incorporation of radioactivity with variation of these two parameters.

Pyridine Nucleotide Requirements. In the work reported previously from this laboratory (Barron, 1966), isocitrate and DPN$^+$ were found to be requisites in the incorporation of acetate into fatty acids by whole mitochondria. Table I shows that this combination can be replaced by DPNH alone in the soluble system; however, there was a consistent small reduction in acetyl-CoA incorporation on omission of TPN$^+$. In the absence of isocitrate, the combination of

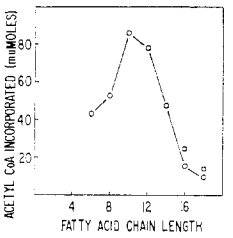

FIGURE 3: The effect of fatty acids of various chain lengths of incorporation of [1-^{14}C]acetyl-CoA into fatty acids. The conditions used were the same as those of Figure 1, except DPNH was substituted for DPN$^+$, and each sample contained 4.5 mg of protein; (⊙) saturated fatty acids; (⊡) Δ^9-monounsaturated fatty acids.

DPNH and TPNH yielded less activity than DPNH alone, suggesting that endogenous pyridine nucleotides were present. The results of testing the charcoal-treated enzyme are seen in Table II. Some activity still remained in the absence of added pyridine nucleotides, but it was apparent that DPNH was required for incorporation of acetate. It was also apparent that DPNH and TPNH together gave a small but consistent increased incorporation of acetate above that obtained with DPNH alone. Both FAD and FMN at 10^{-3} M concentration were found to inhibit the incorporation of acetyl-CoA by 80 and 90%, respectively.

The 20% loss of activity by charcoal treatment has not yet been explained. It could be due to absorption of a cofactor, absorption of a short-chain fatty acid, or the absorption of an enzymatic protein.

Effect of Chain Length. The incorporation of acetyl-CoA could be altered markedly by varying the chain length of the fatty acid added. Figure 3 shows these results. Although 10:0 acid showed the maximum incorporation, it was not normally used as a substrate because of a relatively low boiling point and the attendant problems of handling (both it and its products). As a compromise between low volatility and high incorporation, 14:0 or 16:1[9] was usually used.

Activation of the Substrates. The fact that the CoA derivatives of acetate and long-chain acid were required for activity is shown in Table III. Acetyl-CoA was incorporated four times as rapidly as acetate with ATP and no added CoA. When ATP was deleted, acetyl-CoA incorporation was markedly reduced, indicating that the long-chain acid also had to be activated. No added CoA was required when acetyl-CoA and ATP were used because of the presence of acetyl-CoA deacylase (see below), which apparently released CoA at an adequate rate for the synthesis of the long-chain acyl-CoA. Good activity could also be obtained with acetyl-CoA and long-chain acyl-CoA derivatives in the absence of ATP and CoA (Table IV and Table VI).

The Presence of Malonyl-CoA Decarboxylase and Acyl-CoA Deacylase. The incorporation of significant amounts of [^{14}C]malonyl-CoA into fatty acids occurred only in the

TABLE I: The Requirement for Reduced DPN$^+$.

Test System	mμmoles of Acetate Incorporated into Fatty Acids
Complete[a]	25.2
−Isocitrate	12.0
−DPN$^+$	11.6
−TPN$^+$	23.6
−(Isocitrate, DPN$^+$, TPN$^+$) +DPNH	26.2
−(Isocitrate, DPN$^+$, TPN$^+$) +TPNH	10.9
−(Isocitrate, DPN$^+$, TPN$^+$) +DPNH, +TPNH	20.2

[a] The complete system included 20 μmoles of ATP, 0.12 μmole of CoA, 0.4 μmole each of DPN$^+$ and TPN$^+$, 0.8 μmole of 16:1 potassium salt, 10 μmoles of Mn^{2+}, 140 μmoles of potassium phosphate buffer (pH 7.1), 15 μmoles of isocitrate, 5 μmoles of glutathione, and 1 μmole of [1-^{14}C]-acetyl-CoA (sp act. 2 mCi/mmole), and 4.1 mg of protein. Where indicated, 0.4 μmole of either DPNH or TPNH was added. Final volume 1 ml. Incubation was for 1 hr at 37°.

TABLE II: The Pyridine Nucleotide Requirement for Charcoal-Treated Enzyme.[a]

Test System	mμmoles of Acetate Incorporated into Fatty Acids
Complete (nontreated enzyme)	27.0
Complete (charcoal-treated enzyme)	21.9
−TPNH	18.8
−DPNH	3.9
−DPNH, TPNH	4.1

[a] The complete mixture here was the same as the complete mixture in Table I, with the exception that the DPN$^+$, TPN$^+$, isocitrate combination was replaced by 0.4 μmole each of DPNH and TPNH; 5.0 mg of protein was used per sample. Incubation was for 1 hr.

TABLE III: The Requirement for Activated Substrates.

Elongating Substrate	Incubation Medium[a]	mμmoles of Acetate Incorporated into Fatty Acids
Acetate	Complete	14.1
Acetate	−CoA	6.0
Acetyl-CoA	Complete	26.9
Acetyl-CoA	−CoA	28.7
Acetyl-CoA	−ATP	2.2

[a] The incubation medium was the same as that in Table I except as noted in the table; 5.8 mg of protein was used per sample.

TABLE IV: The Incorporation of Acetyl-CoA and Malonyl-CoA in the Presence of Either 14:0-Acid Plus Its Activating Cofactors Or 14:0-CoA.[a]

Substrate	Elongating Substrate	mμmoles Incorporated into Fatty Acids
14:0-Acid[b]	[1-^{14}C]Acetyl-CoA	36.6
14:0-CoA	[1-^{14}C]Acetyl-CoA	35.7
14:0-Acid[b]	[2-^{14}C]Malonyl-CoA	17.5
14:0-CoA	[2-^{14}C]Malonyl-CoA	2.0

[a] All samples contained 0.4 μmole each of DPNH and TPNH, 0.6 μmole of fatty acid or fatty acyl-CoA, 140 μmoles of potassium phosphate buffer (pH 7.1), 1 μmole of [1-^{14}C]acetyl-CoA or [2-^{14}C]malonyl-CoA (sp act. 2 mCi/mmole), and 5.0 mg of protein. Distilled water was added to a final volume of 1 ml. Incubation was for 15 min. [b] In those samples which contained the fatty acid, the activating cofactors ATP (20 μmoles), CoA (0.12 μmole), and Mn^{2+} (10 μmoles) were substituted for an equivalent volume of distilled water (final volume 1 ml).

presence of activating cofactors (Table IV). It could be shown that essentially no incorporation of malonyl-CoA occurred in the absence of Mn^{2+} (or Mg^{2+}), and it was then suspected that malonyl-CoA decarboxylase was present which required a divalent ion for activity. This was confirmed by incubating [^{14}C]malonyl-CoA with enzyme and Mn^{2+} ion, and at various time intervals adding aliquots to neutralized ethanolic hydroxalamine to stop the reaction and form the hydroxamates. The hydroxamates were separated by paper chromatography and the results are shown in Figure 4. Malonyl-CoA was rapidly decarboxylated to acetyl-CoA in the presence of Mn^{2+}. The half-life of malonyl-CoA was estimated to be 5 min. When acetyl-CoA was incubated with the enzyme ATP and HCO_3^- and Mn^{2+}, it was not possible to demonstrate the formation of malonyl-CoA; however, acetyl hydroxamate and acetate formation were demonstrated. To obtain a better estimate of the acetyl-CoA deacylase activity, the substrate was incubated without added ATP and the half-life of acetyl-CoA was shown to be 10 min.

Long-chain acyl-CoA derivatives were also found to undergo rather rapid hydrolysis. Myristoyl-CoA was incubated with enzyme alone at pH 7.1, and at various time intervals aliquots were treated with $NaBH_4$ in tetrahydrofuran to reduce the thiol ester bonds (Barron and Mooney, 1968). The products were quantitated by gas–liquid partition chromatography and the half-life of the long-chain acyl-CoA derivatives was calculated to be 12 min, a value comparable with that obtained for the acetyl-CoA. The presence of these hydrolases explains why deletion of ATP from the reaction mixtures containing acyl-CoA substrate caused a decrease in acetate incorporation.

The Effect of Avidin. Since the above results showed that malonyl-CoA did not participate in the elongation, it was surprising to find that avidin sometimes inhibited the reaction.

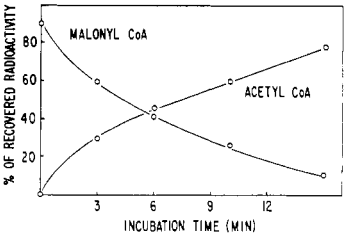

FIGURE 4: The decarboxylation of malonyl-CoA to acetyl-CoA with time. The incubation mixture contained 300 μmoles of ATP, 30 μmoles of Mn^{2+}, 300 μmoles of potassium phosphate buffer, and 14.8 mg of protein in a total volume of 3 ml.

Usually slight or no inhibition was elicited, but occasionally large diminutions in acetate incorporations were noted (40% inhibition on one occasion) when the endogenous acyl-CoA synthetase was utilized for forming myristoyl-CoA. Inhibition was not seen when synthetic acyl-CoA was used. This variability is illustrated in Table V where two different lots of avidin were used. Lot A gave some inhibition at a high concentration of avidin when K-myristate was used, but no inhibition when myristoyl-CoA was used as substrate. With lot B, there was no inhibition when either substrate was used. (Both inhibited malonyl-CoA synthesis as measured by the fatty acid synthetase system.) When inhibition was obtained it was reversible by addition of biotin, but biotin alone stimulated incorporation, so that it is unclear at present how avidin and biotin affect the incorporation of acetate. There was no change in the fatty acids labeled, or the distribution of radioactivity with either avidin inhibition or biotin stimulation.

Elongation of Fatty Acids. After incubation of either myristoyl-CoA or palmitoyl-CoA, DPNH, TPNH, and [1-^{14}C]acetyl-CoA with enzyme, the radioactive fatty acids were isolated by gas–liquid chromatography and the distribution of radioactivity in the various acids determined. Table VI shows that the majority of radioactivity was found in the fatty acids two carbons longer than the added acids.

The isolated acids were decarboxylated and it was found that essentially all the radioactivity had resided in the carboxyl carbons of the acids. For instance, when myristoyl-CoA

TABLE V: Effect of Avidin on Incorporation of Acetyl-CoA.

	% Original Activity		
Substrate[a]	Avidin	Avidin and Biotin	Biotin
A 14:0, K salt	87	107	120
14:0, K salt			119
14:0, CoA ester	101	96	
B 14:0, K salt	99	107	119
14:0, CoA ester	98	108	110

[a] A and B represent experiments with two lots of avidin. The basic reaction conditions used were the same as those in Table IV with [1-^{14}C]acetyl-CoA as substrate. The avidin concentration war 1 mg/ml.

TABLE VI: The Dependency of Chain Length of Acids Labeled on Chain Length of Acid Added.[a]

Added Fatty Acid	% Radioactivity in Products			
	C_{12} Acids	C_{14} Acids	C_{16} Acids	C_{18} Acids
14:0 CoA	0	18.7	76.1	2.0
16:0 CoA	0	3.9	15.4	80.2

[a] The reaction conditions were the same as in Table IV, footnote *a*, with [1-^{14}C]acetyl-CoA as substrate.

was used as the long-chain acid substrate, an average of 97% of the radioactivity found in the C_{16} acids was in the carboxyl carbon.

Discussion

The work presented here confirms the findings reported from Wakil's laboratory concerning a chain elongating system which exists in mitochondria (Harlan and Wakil, 1962, 1963). Further, we have shown that because of contaminating enzymes, acetate, malonyl-CoA, and fatty acids, as well as fatty acyl-CoA and acetyl-CoA, could all, under appropriate conditions, be incorporated into elongated fatty acids.

Acetyl-CoA was shown to be the condensing unit in the elongation process. While carbon atoms of malonyl-CoA could be incorporated, the rate of incorporation was less than that of acetyl-CoA, and only occurred to a significant extent in the presence of divalent ions (Mg^{2+}, Mn^{2+}). A malonyl-CoA decarboxylase in the enzyme preparation was activated by the divalent ions, and it was the resulting acetyl-CoA which was incorporated. It was also not possible to directly demonstrate an acetyl-CoA carboxylase in the preparation, and added ATP was not required for acetyl-CoA incorporation when it and the acyl-CoA ester were used as substrate.

An enigma to the problem of acetyl-CoA *vs.* malonyl-CoA as the condensing unit revolved around the effect of avidin on acetyl-CoA incorporation. When the endogenous acyl-CoA synthetase was utilized with potassium salts of long-chain acids, occasional lots of avidin produced some inhibition of acetyl-CoA incorporation. This inhibition did not occur when the synthetic acyl-CoA esters were used as substrate. This finding remains unexplained, but we concluded that avidin, at least the crude avidin used, must have properties (or impurities) other than that of binding biotin which affects the reaction of fatty acids in this system.

The mechanism of chain elongation by the mitochondrial system seems to be entirely different from the system located in microsomes. Nugteren has shown that malonyl-CoA is the condensing unit and TPNH is the only cofactor necessary for the microsomal system. In the mitochondrial system, acetyl-CoA not only is the condensing unit, but both DPNH and TPNH seem to be required.

The specificity curve obtained for the elongating system shows that fatty acids of 10 to 20 carbons are most efficiently elongated. Substituting the CoA esters for the potassium salts of 14:0 and 16:0 did not affect the amount of incorporation of these fatty acids, so that the curve would appear to apply to the elongating enzymes and not the activating one. However, two other possible modifying factors must be recognized. First, the solubilities of the fatty acid potassium salts may dictate the form of the specificity curve, and, second, the presence of a highly active deacylase which produces the fatty acids from the CoA esters. In the latter case, both the CoA esters and fatty acids would have some equivalency in so far as their ability to participate in elongation. Because of the presence of deacylase, and because the curve obtained closely resembles the activating enzyme activity obtained by Kornberg and Pricer (1953), the specificity curve obtained here may not be that of the elongating enzyme. Purification of the system will be necessary to obtain the information which will aid in the clarification of the problem.

The role of this system in fat metabolism is yet to be elucidated. If the activity curve obtained does apply to the elongating enzymes, they are most active in elongating medium chain acids. Thus, this mitochondrial system may not be the primary system participating in the extension of fatty acids synthesized *de novo*.

Acknowledgment

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Identification of Possible Intermediates in the Mitochondrial Fatty Acid Chain Elongation System*

Edward J. Barron and Larry A. Mooney

ABSTRACT: Chain elongation has previously been described as the addition of a C_2 unit onto preexisting fatty acids. The enzyme system from mitochondria was able to combine acetyl coenzyme A (acetyl-CoA) and long-chain acyl-CoA in the presence of DPNH plus TPNH. In the work presented here, four products from the chain elongation system have been identified. They are 3-hydroxy-, Δ^2-, Δ^3-unsaturated, and saturated fatty acids. Their rates of formation and the influence of cofactors on their formation are used to provide evidence that the 3-hydroxy and Δ^2 acids are intermediates in the elongation. There was no indication of a keto acid as an intermediate. The evidence strongly supports a reaction mechanism whereby the unfavorable energetics for a carbon–carbon fusion between acetyl-CoA and acetyl-CoA is overcome by a rapid reduction requiring DPNH, and producing the 3-hydroxy acid as the first intermediate.

The previous works from this laboratory (Barron, 1966; Mooney and Barron, 1969) have confirmed the findings of Harlan and Wakil (1962, 1963) concerning chain elongation of fatty acids by mitochondrial enzymes. A soluble system prepared from rat liver mitochondrial acetone powder was capable of elongating long-chain acyl-CoA with acetyl-CoA in the presence of DPNH and TPNH.

The condensation of acetyl-CoA and acetyl-CoA to yield a net synthesis of carbon–carbon bonds by a thiolase reaction is not likely since the equilibrium constant of the reaction is in favor of cleavage. Thus the mechanism of chain elongation by the mitochondrial enzymes must involve a modification of the thiolase reaction.

The objectives of this study were to identify the intermediates of the chain elongation reaction, and in conjunction with the cofactor requirements attempt to clarify the mechanism of condensation and the sequence of reactions leading to the saturated acid.

Materials and Methods

Methyl bromoacetate was purchased from Eastman Chemical Co.; myristaldehyde and palmitaldehyde from J. T. Baker; and $NaBH_4$ and $LiAlH_4$ were from Ventron, Metal Hydrides Division.

Gas chromatographic supports and phases were obtained from Analabs, Inc., and were used in either a Model 600 Research Specialties gas chromatograph using a Strontium-90 ionization detector, or a Model 1600 Warner–Chilcott instrument fitted with a flame-ionization detector and an effluent splitter. Samples emerging from the columns were collected on glass wool packed loosely in 0.9 × 4.4 cm glass cartridges held in a Packard gas chromatographic fraction collector, Model 850. With this system, an average of 85% of the radioactivity of injected acids, C_{12} or greater, could be collected from the column effluent if the carrier gas flow rate did not exceed 90 ml/min. In order to collect shorter chain fatty acids effectively, lower gas flow rates were used along with a sample-changer tube holder that allowed cooling of the collection tubes to −5° (salt–ice mix).

The preparation of the enzyme and assay of the enzyme reaction were carried out as previously described (Mooney and Barron, 1970).

The Chemical Synthesis of Fatty Acids. The 3-hydroxy methyl esters of fatty acids were synthesized by the Reformatsky reaction according to Shriner (1942), using the long-chain aldehyde with methyl bromoacetate. Both Δ^2 and Δ^3 acids were obtained by condensation of the long-chain aldehyde with malonic acid. The former was obtained by a Doebner-type procedure using 10% pyridine in acetic acid (Jenny and Grob, 1953). If collidine was used to solubilize the reactants, then Δ^3 acids were produced in good yields (Howton and Davis, 1951). The 3-keto fatty acid esters were obtained by the acetoacetate condensation method of Stallberg-Stenhagen and Stenhagen (1944), except that the acyl chloride was reacted with the sodioethyl acetoacetate in benzene rather than ether. In all syntheses, the products were purified by silicic acid chromatography and/or gas–

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