75, 2520.
Steinberg, G., Slaton, W. H., Jr., Howton, O. R., and Mead, J. F. (1956), *J. Biol. Chem. 220*, 257.
Trams, E. G., and Brady, R. O. (1960), *J. Amer. Chem. Soc. 82*, 2972.
Vandenheuvel, F. A. (1956), *Anal. Chem. 28*, 362.
Vignais, P. V., and Zabin, I. (1958), *Biochim. Biophys. Acta 29*, 263.

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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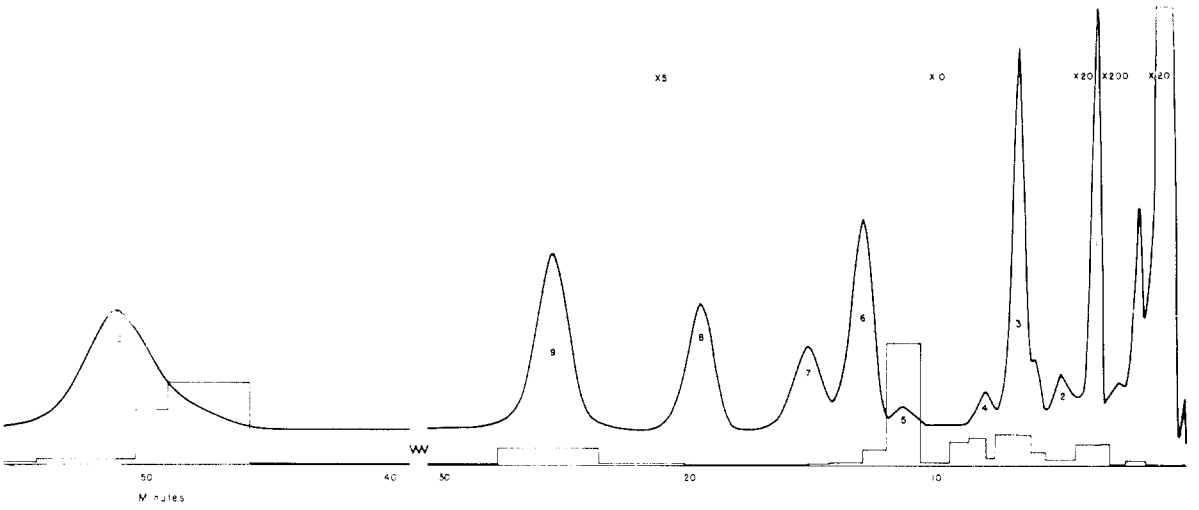

FIGURE 1: The gas–liquid partition chromatography and radioassay of products obtained using 14:1 CoA and [1-^{14}C]acetyl-CoA as substrates. The reaction mixture contained 0.6 μmole of 14:0 CoA, 1.0 μmole of [1-^{14}C]acetyl-CoA, 0.35 μmole each of DPNH and TPNH, 5.6 mg of protein, and 140 μmoles of potassium phosphate buffer (pH 7.1) all in 1-ml final volume. Incubation was at 37° for 15 min. Areas of radioactivity are indicated by the bar graph. Peaks 1, 3, and 6 are the saturated methyl esters of 14-, 16-, and 18-carbon fatty acids, respectively.

liquid partition chromatography.[1] The coenzyme A esters of the 16:1[2] and 3-OH 16:0 acids[2] were prepared by the mixed-anhydride method of Wieland and Rueff (1953), as modified by Goldman and Vagelos (1961).

Structure Proofs. The compounds synthesized as outlined above were identified by their gas chromatographic behavior before and after the chemical modifications described below. The gas–liquid partition chromatography retention data obtained were compared with those of commercially available compounds, as well as with data found in the literature. Infrared spectra of the samples in KBr disks were obtained with a Perkin-Elmer Model 700 instrument fitted with a beam condenser.

Fatty acids were esterified 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), except that methanol was used as a solvent and the reaction time was for 1 hr. Thin-layer chromatographic separations of fatty esters were carried out utilizing Eastman Chromagram sheets and hexane–ethyl ether–acetic acid (80:20:1). $LiAlH_4$ was utilized to reduce *O*-acyl esters, using ether under reflux for 45 min. After destroying excess $LiAlH_4$ with methanol, the solution was acidified and the alcohols extracted with ether. The Von Rudloff (1956) oxidation procedure was employed to identify the location of double bonds in the unsaturated compounds.

The van Slyke oxidation as described by Davidoff and Korn (1964), was applied to the characterization of the 3-hydroxy fatty acids.

Acetylation of hydroxyl groups was accomplished by drying the sample in a test tube followed by the addition of 0.5 ml of acetic anhydride and 5 mg of *p*-toluenesulfonic acid. After allowing the mixture to sit for 10 min at room temperature, ether was added and the mixture washed with four aliquots of distilled water (2 ml of each).

Products of the Elongation Reaction. Initially the collections from the effluent of the gas chromatograph were made on the basis of the mass response, as seen on the strip-chart recorder. That is, the effluent corresponding to each peak was collected into a separate cartridge. However, this procedure soon proved to be unsatisfactory since most of the products had large amounts of radioactivity but little mass, and did not chromatograph with the normal esters whose mass was detected. Subsequently all peaks were fractionated into two parts and at least one separate collection was made between mass peaks. Figures 1 and 2 show the chromatograms with associated radioactivity of the methyl esters of the fatty acids isolated from reactions in which 14:0 CoA and 16:0 CoA, respectively, were used as substrates along with [1-^{14}C]-acetyl-CoA, DPNH, and TPNH. In Figure 1, all the mass peaks except 5 and 9 are from endogenous acids, and peak 1 is primarily from 14:0 CoA. As will be described in following sections, the acids containing radioactivity were found to be: 14:0 in peak 1, in peak 2, 16:0; on the downward side of peak 4, 16:1[3]; in peak 5, 16:1[2]; in peak 9, 3-OH 14:0, and the radioactivity in the upward side of peak 10 was in 3-OH 16:0. In Figure 2, the compounds containing radioactivity have been identified as methyl esters of 14:0 and 16:0, 16:1[3], 16:1[2], and 18:0; and that in the downward side of peak 7 as 18:1[3]; on the downward side of peak 8 as 18:1[2]; peak 9 at 40.5 min was 3-OH 16:0, and that at 76.5 min was 3-OH 18:0.

[1] Glass gas–liquid chromatographic columns were found to be best for separation and purification of most of these compounds. The 3-OH fatty acids in particular showed considerable degradation on stainless steel columns at 180° (E. J. Barron and L. A. Mooney, unpublished data).

[2] Abbreviation used is: RRT, relative retention time (always relative to the saturated ester of the same carbon length). The shorthand abbreviations of Ahrens *et al.* (1959) is used to identify fatty acids. The first numeral designates the number of carbons and the one after the colon the number of double bonds. Superscript numerals are sometimes given to identify the position of the double bonds, *i.e.*, myristic, palmitic, and oleic acids are represented by 14:0, 16:0, and 18:1[9], respectively.

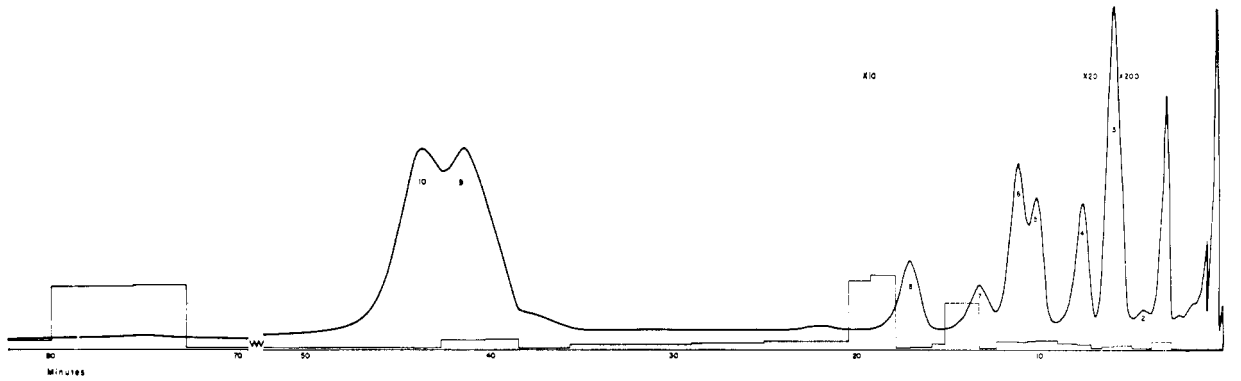

FIGURE 2: The gas–liquid partition chromatography and radioassay of products obtained using 16:0 CoA and [1-^{14}C]acetyl-CoA as substrates. Reaction conditions were the same as given in Figure 1. Peaks 1, 3, and 6 are the methyl esters of 14-, 16-, and 18-carbon fatty acids, respectively.

These chromatograms illustrate that the majority of the [1-^{14}C]acetyl-CoA was incorporated into compounds two carbons longer than the added long-chain acid. Nevertheless, some radioactivity was found in acids of the same chain length as the added fatty acid, and some in acids two carbons shorter than the added acid.

Hydroxy Fatty Acid Esters. The gas chromatographic retention times of peaks 9 and 10 corresponded closely with those of the synthetic methyl 3-hydroxy fatty esters of 14:0 and 16:0 (RRT = 6.60), and in addition were in good agreement with the relative retention times given by Davidoff and Korn (1964), and Tulloch (1964) for the 3-hydroxy esters. Again, after acetylation, good correspondence of their retention times to that of the standard acetoxy esters was obtained (RRT = 6.14). Also, the acids corresponding to peaks 9 and 10 could be separated from the others by thin-layer chromatography, which allowed separation of larger quantities.

Final proof that these compounds were indeed the methyl 3-hydroxy fatty esters was obtained by oxidizing the isolated hydroxy acids by the Van Slyke procedure.[3] Of the total radioactivity, 72% could be trapped as CO_2 in phenethylamine. Nonradioactive 2-pentadecanone was identified as the product on oxidation of the 3-OH 16:0 acid, and 2-tridecanone was obtained from the 3-OH 14:0.

Δ^2-Unsaturated Fatty Acids. Peak 5 of Figure 1 and the radioactive compound on the downward side of peak 8 in Figure 2 were tentatively identified as being *trans* Δ^2 esters since their gas–liquid partition chromatography retention times corresponded with that of the synthetic compounds (RRT = 1.63). The presence of the double bond was confirmed by catalytic hydrogenation of the isolated compounds. The radioactivity and the mass were subsequently found to correspond with 16:0 and 18:0 esters. Oxidation (Von Rudloff, 1956) of the unsaturated compounds produced the acids 14:0 and 16:0, and allowed the collection of 70% of the radioactivity in CO_2 traps. The *trans* conformation was assigned those compounds since the *cis* ester had been reported to have a much shorter retention time than the *trans* (Lennarz *et al.*, 1962; Davidoff and Korn, 1964). This was confirmed here by overnight ultraviolet irradiation of the synthetic Δ^2 ester in acetone. A new compound was then demonstrated by gas–liquid partition chromatography which had a RRT of 1.04 and constituted 38% of the sample. Oxidative cleavage of this compound confirmed the location of the double bond as being in the 2 position.

Δ^3-Unsaturated Fatty Acids. The analyses of samples which were believed to be the Δ^3 esters (the radioactive compounds on the downward side of peak 4, Figure 1, and the downward side of peak 7, Figure 2) were hampered by the gross contamination of these fractions with the Δ^9 compounds (RRT = 1.27 and 1.18 for the Δ^3 and Δ^9, respectively). Hydrogenation caused their retention times to decrease to that of the saturated esters, while oxidation of the double bond permitted the trapping of 60–70% of the radioactivity in CO_2 traps. Unfortunately, these compounds were not present in sufficient quantities to allow the identification of the monocarboxylic acid product formed on oxidation. However, the ability to collect the radioactivity from the carbonyl carbon indicated that these were Δ^2- or Δ^3-unsaturated compounds. Furthermore, the RRT of 1.27 (estimated from the radioactivity) was sufficiently different from both the Δ^9 (RRT = 1.18) and the *cis*-Δ^2 (RRT = 1.04) to enable us to assign the double bond to the 3 position.

Freedman and Becker (1951) reported that $LiAlH_4$ partially reduced the double bond of Δ^2-aliphatic acids; however, we have found that under the conditions used, this agent completely hydrogenates Δ^2 acids to the saturated alcohols. Since the Δ^3 acids were not reduced, this procedure has been used to confirm their structure. Duplicate standard samples were treated with $LiAlH_4$. One of the samples was subsequently gas chromatographed, the other treated with KIO_4–$KMnO_4$ (Von Rudloff, 1956) and then gas chromatographed. The products from the standard Δ^2 esters chromatographed with standard alcohols of the same chain length, even after treatment with the oxidizing agent, whereas the products of standard Δ^3 esters migrated more slowly and were oxidized to the acid of three less carbons. The radioactivities of the unknown samples were found after $LiAlH_4$ treatment to

[3] Van Slyke oxidation of 3-hydroxy acids produces the 2-methyl ketones with one less carbon, whereas oxidation of methyl esters yields fatty acids with two less carbon atoms (E. J. Barron and L. A. Mooney, 1968, unpublished data).

chromatograph with the reduced standard Δ^3 esters (Δ^3 alcohols) and were lost upon KIO_4–$KMnO_4$ oxidation.

Using synthetic 3-OH 16:0 CoA with 1 mg of enzyme protein/ml (to minimize endogenous acid), a sufficient amount of 16:1[3] acid was isolated for further analysis. The Δ^3 acid obtained was purified by two passes through a gas–liquid chromatograph with the fractions being taken so as to eliminate 16:1[9] and 16:1[2] acids. When this acid was oxidized with permanganate–periodate, tridecanoic acid was isolated as the primary product (>90%). The infrared spectra showed that the double bond was in the *cis* configuration. Besides the absorption peaks associated with double bonds at approximately 3000 and 1645 cm^{-1}, medium absorption maximum at 1425 and 705 representing the CH rock and CH wag, respectively, of a *cis* double bond were present. Thus, these compounds (RRT = 1.27) have been identified as methyl esters of *cis*-Δ^3-fatty acids.

Davidoff and Korn (1964) reported that the hydrolysis of Δ^2 methyl esters with 0.5 M NaOH in 50% methanol for 2 hr caused partial isomerization, *via* the methoxy intermediates, to the Δ^3 acids. Similar results were found here using 1 N NaOH in 50% methanol at 65° for 30 min. Approximately 10% of the Δ^2 methyl ester was converted into the 3-methoxy acid (RRT 2.31 for its ester relative to the straight-chain methyl ester) and 2% to the Δ^3 acid. The Δ^2-acyl-CoA ester was likewise converted into the 3-methoxy acid in about the same yield, but in this case no formation of Δ^3 acids could be demonstrated. The conversion of Δ^2 acyl-CoA into the 3-methoxy compound was also seen at 38° after 10 min, conditions which left the Δ^2 methyl ester unchanged. In distinction to both the methyl ester and CoA ester, the free Δ^2 acid was unaffected after treatment for 30 min at 65° with methanolic NaOH or with sodium methoxide.

These differences between the Δ^2 acyl-CoA ester and methyl ester in the formation of the 3-methoxy acid under different reaction conditions and the lack of formation of the Δ^3 acid from the CoA ester hydrolysis are undoubtedly related to the fact that the divalent sulfur of the CoA ester has little tendency to form double bonds, making the resonance structures involving positively charged sulfur unlikely (Lynen, 1961). The lack of formation of the Δ^3 acid from the Δ^2 acyl-CoA ester could then be explained by the electron delocalization being more directed toward the carboxyl group in the thioester than in the case of the *O*-acyl ester.

The inability to experimentally show that Δ^3 acids arose on hydrolysis of Δ^2 acyl-CoA esters in methanolic base, and the fact that the biosynthesized acids were present only as the acyl-CoA esters or free fatty acids indicated that the Δ^3 acids isolated from the enzyme reaction were not artifacts. Two experiments were carried out to further substantiate this. The biosynthesized CoA esters were isolated by perchloric acid precipitation from 12-min reaction mixtures in which [1-^{14}C]acetyl-CoA and 14:0 CoA were used as substrates and DPNH and TPNH as cofactors. The samples were then hydrolyzed at 65° for 15 min in 0.05 N KOH made up in the following solvents: 50% ethanol, 50% methanol, 33% tetrahydrofuran, or in *t*-butyl alcohol which was saturated with 15 N aqueous KOH. The percentage of radioactivity in the Δ^3 16:0 acid, found by gas chromatography of the methyl esters, was 8.5, 9.4, 8.9, 9.4, and 9.5, respectively. In the sample hydrolyzed in methanol, there was also 9.6% of the radioactivity in the methoxy C_{16} acids, and in that hydrolyzed in ethanol 10.4% was found in the ethoxy-C_{16} acid. Finally, if the reaction was incubated for an extended period of time (45 min) so that the endogenous acyl-CoA deacylase had hydrolyzed most of the radioactive thiol esters (60% free acids), the biosynthesized free acids could be isolated without hydrolysis (see section on Derivative Form of the Elongated Products). The samples were esterified and separated by gas–liquid chromatography and in these experiments approximately 15% of the radioactivity was found in the Δ^3 acid. This relatively large amount of Δ^3 acid was due to the longer incubation period. Thus, the formation of the same relative amount of the Δ^3 acid, using different hydrolyzing media, and the detection of Δ^3 acids without hydrolysis indicate that these were not artifacts of isolation.

Keto Acids. The 3-keto acids have not as yet been demonstrated to be intermediates in this enzyme system. The conditions used to isolate fatty acids are known to decarboxylate 3-keto acids (Davidoff and Korn, 1964; Nugteren, 1965) and, indeed, synthetic 3-keto 16:0 carried through our procedure showed complete conversion into 2-pentadecanone. The combinations, [2-^{14}C]acetate plus 14:0 and acetate plus [1-^{14}C]-14:0, were both used for substrates. The rationale was that if the keto acid was formed and subsequently decarboxylated, the product 2-pentadecanone would still be radioactive and thus identifiable. In both cases nonradioactive 2-pentadecanone was added in the samples after stopping the enzymatic reaction with methanolic KOH. The saponified samples were extracted while still alkaline, a portion of each sample was gas chromatographed, and in neither case was the isolated 2-pentadecanone found to contain radioactivity.

The keto trapping agents, hydrazine and semicarbazide, occasionally produced a small stimulation of acetate incorporation at low concentrations, but otherwise had no effect at concentrations up to 2×10^{-2} M. Girard T reagent had no effect. The samples from the experiments were hydrolyzed in acid and base and the distribution of counts as determined by gas chromatography were the same as the controls.

Derivative Form of the Elongated Products. By separating products of the reaction into acyl-CoA derivatives and lipid fractions (free acids plus *O*-acyl esters) by the method of Porter and Long (1958), the radioactive acids in the acyl-CoA fraction were shown to decrease, while those in the lipid fraction increased with time (Table I). Because the radioactive acids in the lipid fraction could exist as free acids or esters, the composition of this fraction was investigated. It was further separated into a neutral lipid and phospholipid fractions on a 1-g silicic acid column prepared by the method of Barron and Hanahan (1958), whereby the neutral lipids were eluted with 30 ml of $CHCl_3$, and the phospholipids with 50 ml of chloroform–methanol (1:9). The neutral lipid fraction contained 70–80% of the radioactivity. The neutral lipids were partitioned between hexane and 0.05 N NaOH in 50% ethanol according to Borgstrom (1952), and essentially 100% of the radioactivity was found in the free fatty acid fraction. The phospholipid fraction was directly esterified with diazomethane and gas chromatographed. The fatty acid methyl ester contained 85–90% of the counts when corrections were made for collection efficiency. Furthermore, approximately 90% of this radioactivity was in 3-OH acid methyl esters; the remainder was in Δ^2 and Δ^3 acids

TABLE I: The Inverse Relationship of [1-^{14}C]Acetyl-CoA Found in Lipid and CoA Ester Fractions as a Function of Time.[a]

Incubation Time (min)	% Radioactivity in Fractions	
	Lipids	CoA Esters
5	18	82
10	23	77
15	36	66
30	43	57
45	60	40

[a] The reaction conditions were the same as in Figure 1.

TABLE II: The Distribution of Radioactive Fatty Acids in Various Lipid Fractions after Different Reaction Times.[a]

	10-min % Radioactivity	20-min % Radioactivity
Acyl-CoA	79	58
Lipids and free fatty acids	21	42
Neutral lipid fractions	70	82
Glycerides	2	1
Free fatty acids	98	99
Phospholipid fractions	30	18
Phospholipids	None[b]	

[a] The concentrations of the reactants were the same as described in Figure 1. The final volume was 3 ml. [b] See text.

(Table II). Because esterification with diazomethane, using the conditions of Schlenk and Gellerman (1960), does not catalyze transesterification, essentially all the radioactive acids in this fraction must have existed as free fatty acids. The polarity of their hydroxy groups caused them not to be eluted from the silicic acid column with the other fatty acids.

Thus, with this enzyme preparation, the labeled fatty acids appeared as either the coenzyme A esters or as free fatty acids.

Reversal of Reaction. Although the majority of the incorporated [1-^{14}C]acetyl-CoA was found in the fatty acids two carbon atoms longer than the added long-chain acid, some of the radioactivity was also found in acids the same chain length as the added acid and sometimes in the acids two carbon atoms shorter. This is illustrated in Table III where the Δ^2, Δ^3, 3-OH, and saturated acids of each chain length have been added together to yield the total percentage. (The acids obtained after the addition of potassium 16:1^9 were all of the unsaturated series.) There were relatively more counts in the fatty acids two carbon atoms longer than the added acid and less in the shorter acids if the acyl-CoA derivatives were added directly, than if the endogenous long-chain acyl-CoA synthetase was utilized to form the CoA derivatives. One reason for this was, of course, that in the latter case the endogenous acids could also participate in the reaction.

The endogenous free fatty acids in the enzyme preparation were isolated by a procedure similar to that described in the section on product derivatives (*i.e.*, a blank reaction mixture was extracted with chloroform–methanol, and then carried through the isolation procedure). The free fatty acids were quantitated by gas–liquid chromatography of their methyl esters and comparison with a standard methyl ester preparation (C-120 from Applied Science). The total amount of endogenous free fatty acids present in the amount of enzyme preparation used for a 1-ml reaction (6-mg of protein) was approximately 0.09 μmole. Table IV gives the relative percentage of each fatty acid in the enzyme preparation and the percentage of radioactivity found in the fatty acids of each chain length when the endogenous acids were used as the long-chain substrate.

Further evidence for the reversibility of the chain elongation reaction was obtained by several means. Uniformly labeled 16:0 acid was used as the substrate with ATP, CoA, Mn^{2+}, DPN, and TPN as cofactors. After incubation for 30 min, the samples were hydrolyzed and the acids isolated. Approximately 5% of the radioactivity was now found in 14:0 acid (Table IV). Also, using [1-^{14}C]-14:0 acid as the substrate under the same conditions, 3–4% of the radioactivity could not be accounted for in the long-chain acid fraction. By treating similar reaction mixtures with hydroxalamine and isolating the short-chain hydroxamates and ammonium salts, it was possible to recover the radioactivity in this fraction and to demonstrate acetyl hydroxamate and ammonium acetate by paper chromatography.

Table V shows the effect of adding DPN and TPN to the reaction. The addition of DPN and TPN along with DPNH

TABLE III: The Effect of Type of Long-Chain Acid Derivatives Added on Distribution of Radioactive Products.[a]

Added Fatty Acid	% Radioactivity in Products			
	C-12 Acids	C-14 Acids	C-16 Acids	C-18 Acids
14:0-K salt	0.5	29.4	60.8	5.7
14:0 CoA[b]	0	18.7	76.1	2.0
16:0-K salt	0	14.4	33.2	46.6
16:0 CoA	0	3.9	15.4	80.2
16:1^9-K salt	0	5.6	23.3	63.4
U-^{14}C 16:0-K salt	0.1	4.7	93.2	0.4

[a] The reaction mixture when the potassium salts were used contained 20 μmoles of ATP, 0.12 μmole of CoA, 0.4 μmole of DPNH, 0.4 μmole of TPNH, 10 μmoles of Mn^{2+}, 0.6 μmole of fatty acid, 1 μmole of [1-^{14}C]acetyl-CoA (sp act. 2 mCi/mmole), 140 μmoles of potassium phosphate buffer, pH 7.1, and 5.9 mg of protein. [b] When the long-chain acyl-CoA compounds were used the reaction was the same except ATP, CoA, and Mn^{2+} were not added. The total volume was 1 ml and the incubation time was 30 min. With [U-^{14}C]-16:0 acid as substrate, no acetyl-CoA was added and 0.4 μmole each of DPN and TPN was added in place of DPNH and TPNH.

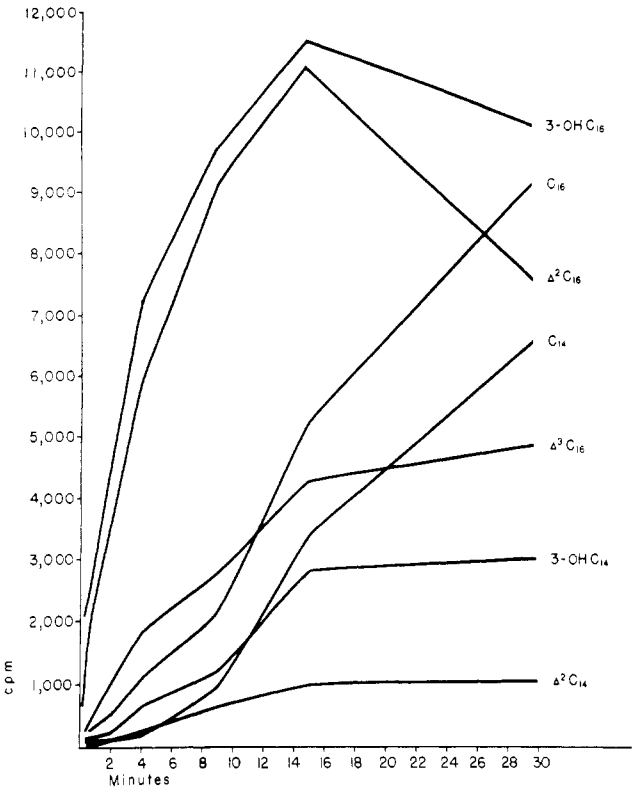

FIGURE 3: The rates of appearance of ^{14}C into fatty acid products from [1-^{14}C]acetyl-CoA plus 14:0 CoA. Reaction conditions were the same as those described in Figure 1.

and TPNH inhibited the incorporation of acetyl-CoA into the long-chain fatty acids, while the amount of radioactivity incorporated into 14:0 acid increased. Both of these would be expected to occur if the reaction were reversible.

The rate of incorporation of [1-^{14}C]acetyl-CoA into fatty acids 14 carbons in length with 14:0 CoA as the substrate is also shown in Figure 3. As might be expected, the rates of formation were somewhat different than that of the elongation reaction since the reverse and forward reactions must occur before labeled compounds appear. Also, the rate of formation of DPN and TPN would be dependent on the rate of reduction of the chain elongation intermediates. In these experiments the Δ^3 14:1 acid was either not formed or at least not in sufficient amounts to be detected.

TABLE IV: The Relative Per Cent of Endogenous Fatty Acids in Enzyme Preparation and Relative Per Cent Radioactivity in Products When They Were the Substrate.[a]

Acid	Endogenous FFA[b] (Mass %)	Product Radioactivity (%)
12:0	~1	C-12 acids 1.6
14:0	10.5	C-14 acids 12.9
14:1[9]	trace	C-16 acids 38.3
16:0	28.6	C-18 acids 32.3
16:1[9]	3.2	C-20 acids 9.4
18:0	29.7	
18:1[9]	16.6	
18:2[9,12]	6.8	
20:4[5,8,11,14]	4.5	

[a] Reaction conditions were the same as in Table II, footnote *a*, except no long-chain acids were added. Reaction time was 30 min. [b] FFA = free fatty acid.

TABLE V: The Effect of DPN and TPN on Reaction.[a]

Additions	μmoles of Acetyl-CoA Incorporated	% Radioactivity in Isolated Acids	
		C-14 Acids	C-16 Acids
Complete None	37	17	71
Complete DPN, TPN, 1.4×10^{-4} M each	33		
Complete DPN, TPN, 2.7×10^{-4} M each	31	30	58
Complete DPN, TPN, 4×10^{-4} M each	23		
Complete DPN, TPN, 8×10^{-4} M each	16	33	54
Complete TPN 4×10^{-4} M	27	23	66
Complete DPN 4×10^{-4} M	27	24	65

[a] The reaction mixture was the same as in Figure 1.

Reaction Time Course. Figure 3 shows the time course for the synthesis of radioactive compounds[4] when 14:0 CoA [1-^{14}C]acetyl-CoA were used as substrates with DPNH and TPNH as the reductants. The compounds formed most rapidly was 3-OH-16:0 with the rate of synthesis of the 16:2 closely paralleling its formation. Initially the next most rapidly formed compound was the 16:1[3] up to about 10 min. During this time its rate of synthesis was about one-third that of the 16:1[2]. After approximately 10 min the rate of synthesis of the saturated 16:0 exceeded that of the 16:1[3] whose rate of formation leveled off. These data indicate that the 16:1[2] arises from the 3-OH-16:O, and suggests that the 16:1[3] also arises from the 3-OH-16:0 acid. Nevertheless, these types of data do not exclude the direct isomerization of the Δ^2 acid to the Δ^3 acid. Also, the saturated 16:0 acid probably arises from the 16:1[2] acid, although if the turnover rate of the Δ^3 was high it too could be the percursor of the saturated acid.

The effect of anaerobic conditions on the synthesis of the acids was tested. The reactions were carried out in Thurnberg tubes which were evacuated and filled with N_2 three times before

[4] Obviously we are speaking here of acetate incorporation and not specific activity, but since these compounds do not normally occur and are not present in our preparations in detectable amounts, the specific activity of these intermediates will be the same as the acetate added. Therefore the acetate incorporated is assumed to be an accurate measure of the specific activity.

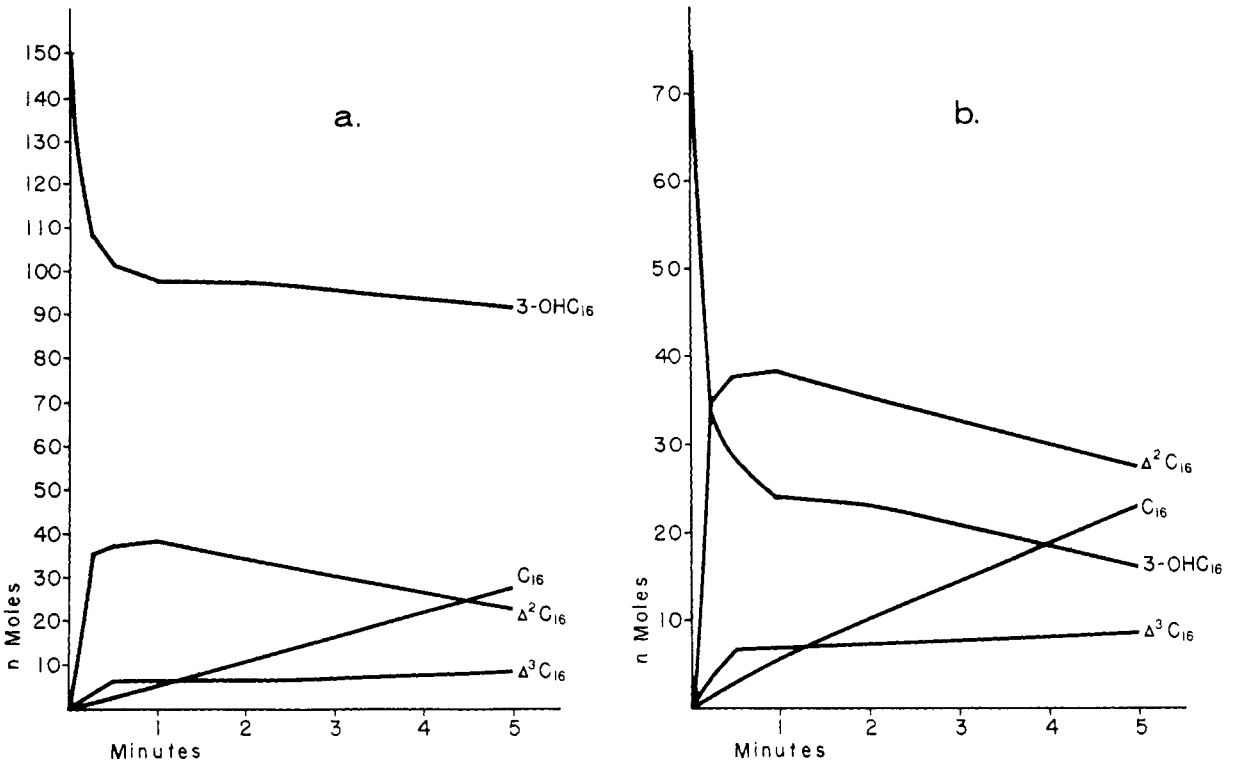

FIGURE 4: The conversion of synthetic 3-OH-16:0 CoA into other 16 carbon acids in the presence of TPNH. The reaction mixture contained 0.15 μmole of 3-OH-16:0, 0.4 μmole of TPNH, 1.7 mg of protein, and 140 μmoles of potassium phosphate buffer (pH 7.1) in 1-ml final volume; incubation at 37°. Results are plotted considering all the substrates as being enzymatically active (a) and 50% as being active (b).

incubation was started. The amount of acetyl-CoA incorporated and the relative percentage of each of the labeled acids were the same.

Testing of Intermediates. The coenzyme A esters of the Δ^2-16:0 and the 3-OH-16:0 acid were incubated with enzyme and TPNH or DPNH at pH 7.1 to assess their participation as intermediates in the chain elongation reaction. The interconversions were large enough in these reactions to allow the mass analysis of the products by gas–liquid chromatography. Figure 4a shows the results obtained with 3-OH-16:0 CoA ester when TPNH was used as a cofactor. The substrate was converted into the Δ^2, Δ^3, and saturated 16:0 acid. If it is assumed that only 50% of the 3-OH acid is the correct stereoisomer for the dehydratase and the results plotted accordingly, Figure 4b is obtained. When plotted in this manner, a greater conversion into the Δ^2 acid and other products was apparent. Figure 5 shows that all the expected compounds can also arise from 16:1[2] CoA. The results obtained with the Δ^2 acyl-CoA suggested that the assumption of approximately 50% of the 3-OH acyl-CoA was utilizable by the hydratase was reasonable. However, even with this assumption, the results of the two experiments are not in very good agreement as regards the relative ratios of Δ^2 and 3-OH acids in relation to time and the rate of saturated acid synthesis.

The reasons for the difference in experimental results which were obtained repeatedly is not clear. The problem with the different Δ^2 acid to 3-OH acid ratios could be due to the wrong assumption about the amount of active stereoisomer present in 3-OH acyl-CoA, or that equilibrium was not obtained in either experiment. The slower rate of synthesis of the Δ^3 acid with the Δ^2 acyl-CoA substrate could readily be explained by the 3-OH acid being its precursor. In turn, the slower rate of synthesis of the saturated acid would be obtained if the Δ^3 acid was its precursor. Perhaps the simplest explanation for the apparent smaller conversion of the Δ^2 acyl-CoA to the other components would be afforded by postulating the presence of an inhibitor in this synthetic substrate.

The data obtained with the 3-OH acyl-CoA suggests that the Δ^2 acid is the precursor of the saturated acid; with TPNH as the cofactor, the formation of the saturated acid roughly parallels the decline of the Δ^2 acid after 1 min. Furthermore, with DPNH as cofactor, there is essentially no saturated fatty acid formed, and after the first 30-sec reaction time the concentration of Δ^2 acid remains reasonably constant (Figure 6). The rate of formation of the Δ^3 acid is approximately the same in both experiments, while if it was the precursor of the saturated acids its accumulation would be expected in the absence of TPNH.

These experiments were not helpful as regards the origin of the Δ^3 acid.

Discussion

The 3-OH and Δ^2 acids appear to be intermediates in the chain elongation reaction. These compounds and the saturated acid, all two carbons longer than the added acyl-CoA ester, were the major radioactive compounds formed when radioactive acetyl-CoA and an acyl-CoA were incubated with

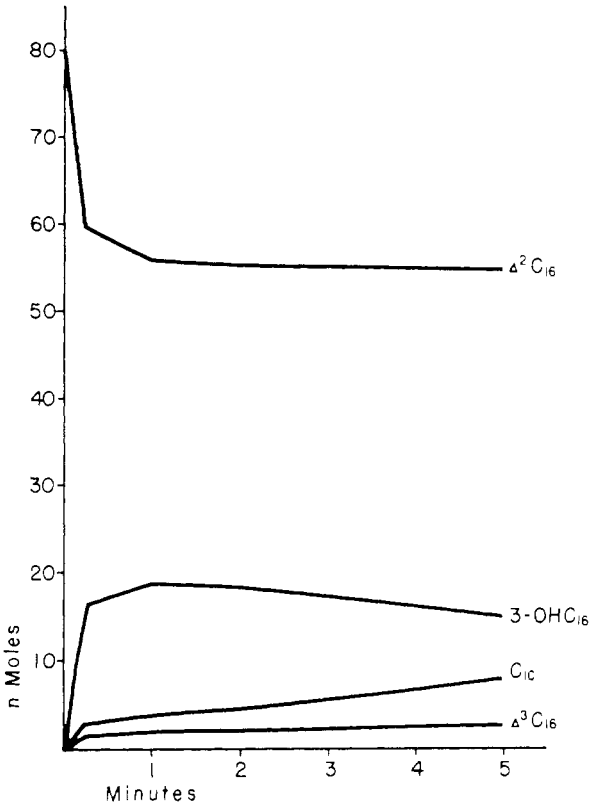

FIGURE 5: The conversion synthetic 16:1[2] CoA into other 16-carbon acids in the presence of TPNH. Conditions were the same as in Figure 4 except 0.080 μmole of 16:1[2] CoA was used as substrate.

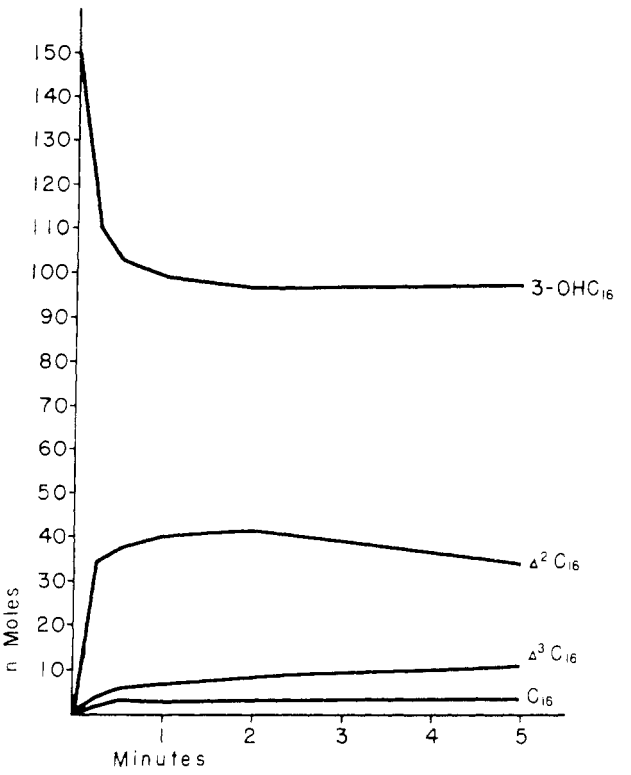

FIGURE 6: The conversion of synthetics 3-OH-16:0 CoA into other 16-carbon acids in the presence of DPNH. Reaction conditions were the same as in Figure 4 except for the substitution of DPNH.

DPNH and TPNH. Furthermore, when either the 3-OH acyl-CoA or Δ^2 acyl-CoA was used as substrate, the other was formed along with the saturated acid. The 3-OH acid and the Δ^2 acid were readily interconvertible with the equilibrium favoring the formation of the Δ^2 acid. The kinetic data indicated that the 3-OH acid was formed first and it was converted into the Δ^2 acid which was then reduced to the saturated acid. In all the experiments, however, a Δ^3 acid was also synthesized. Its role in the sequence of chain-elongation reactions is not clear. The results of the time course studies on acetyl-CoA incorporation would indicate that it is also produced by dehydration of the 3-OH acid. The experiment using the 3-OH or Δ^2 acyl-CoA esters on the initial substrate was of little value in delineating its origin. Thus, the evidence is not sufficient to rule out direct isomerization of the Δ^2 acid to the Δ^3 acid. Also, while the kinetic data is strongly in favor of the Δ^2 acid being the precursor of the saturated acid, it would be possible for the Δ^3 to be the substrate reduced to the saturated acid if its turnover rate was high enough. It is also possible that the Δ^3, as well as the Δ^2 acid, can be reduced to the saturated acid.

Davidoff and Korn (1964) have reported on the conversion of long-chain saturated acids into their Δ^2, Δ^3, and 3-OH acids by enzymes isolated from subcellular particles of a cellular slime mold. Their evidence suggested that saturated acid was converted into the *trans* Δ^2 acid and the *cis* or *trans* Δ^3 acid, and the *trans* Δ^2 acid was the precursor of the D-(−)-3-OH acid. The reversible isomerization of the Δ^2 to the Δ^3 acid occurred apparently without the hydroxy acid being an intermediate. Since only the interconversions were demonstrated, the authors were not certain what role these reactions had in the metabolism of the organism. By process of elimination, they did conclude that they might be participants in a chain elongation process. However, the role that Δ^3 acid might have in chain elongation or perhaps the synthesis of monounsaturated acids was unclear.

While the reactions described in this paper can be ascribed to chain elongation, the same problem exists as regards the role of the Δ^3 acid. The ability to synthesize Δ^3 acids by liver mitochondrial enzymes would appear to confer upon the organ the potential of synthesizing monounsaturated acids *de novo* in a manner similar to some bacteria (Scheuerbrandt *et al.*, 1961). This pathway has not yet been demonstrated in mammals and it is generally considered that mammals synthesize monounsaturated fatty acids only by dehydrogenation of performed saturated acids (Erwin and Bloch, 1964). The synthesis of the Δ^3 acid could be a vestigial part of a mechanism in mammals whereby in evolutionary times synthesis *de novo* of monounsaturated acids did occur. This would imply that these organisms now reduce Δ^3 bonds or the Δ^3 acid cannot be elongated. No evidence was found that the Δ^3 acid formed during the reaction was elongated, but its elongated product may have been present in small amounts and its detection missed on oxidative cleavage of the monounsaturated fractions. There is also no evidence in the literature that a Δ^3 bond is retained in polyunsaturated acids synthesized in mammals. It would thus appear that Δ^3 bonds are reduced in mammals.

The enzyme reactions described were reversible. This

was shown by the rapid exchange of the carboxyl-terminal C_6 unit of the added acid with radioactive acetyl-CoA. Also, net shortening of the added acid was shown by the formation of a small amount of radioactive 14:0 acid from uniformly labeled 16:0 acid in the presence of DPN and TPN. While reversibility does occur, as readily demonstrated by the exchange reaction, the enzyme's function is not degradative in nature since the *net* shortening of fatty acid substrates is demonstrable to only a limited degree. The exchange reaction also occurs in intact mitochondria (Barron, 1966). The possibility of labeling fatty acids by this type of exchange reaction reemphasizes that care must be taken in describing various types of saturated or unsaturated fatty acid synthesis from radioactive acetate, without careful degradative studies.

In these experiments the radioactive fatty acids formed were found either as the CoA derivatives or as free fatty acids, and were incorporated into complex lipids to a very small extent, if at all. Presumably, all the labeled fatty acids formed would be found in the CoA ester form if a deacylase were not present (Mooney and Barron, 1970).

Since acetyl-CoA is the condensing unit for chain elongation, the condensation reaction is most probably a reversal of a thiolase-type reaction. The equilibrium of the thiolase reaction is in favor of cleavage and, as discussed by other investigators (Lynen, 1961), in order for net condensation to take place, an effective reductive process would be necessary. With this mitochondrial system, no significant incorporation of acetyl-CoA occurs in the absence of DPNH (after removal of endogenous nucleotides by charcoal treatment) (Mooney and Barron, 1970), indicating that a reductive process was associated with the condensation reaction. This necessary coupling of reduction with the condensation probably explains the inability to demonstrate a keto acid intermediate, since it would have only a transitory existence. Thus, all the data supports the concept that the condensation of the long-chain acyl-CoA and acyl-CoA by this mitochondrial system is a thiolase-type reaction in which a very rapid, reductive process occurs with the fusion of the reactants.

The experimental data support the following sequences of reaction.

$$RCH_2\overset{O}{\overset{\|}{C}}SCoA + CH_3\overset{O}{\overset{\|}{C}}{-}SCoA \rightleftharpoons (RCH_2\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}SCoA) \quad (1)$$

$$(RCH_2\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}SCoA) + DPNH + H^+ \rightleftharpoons RCH_2\overset{OH}{\overset{|}{C}}HCH_2\overset{O}{\overset{\|}{C}}SCoA + DPN^+ \quad (2)$$

$$RCH_2\overset{OH}{\overset{|}{C}}HCH_2\overset{O}{\overset{\|}{C}}SCoA \rightleftharpoons RCH_2CH{=}CH\overset{O}{\overset{\|}{C}}SCoA + RCH{=}CHCH_2\overset{O}{\overset{\|}{C}}SCoA \quad (3)$$

$$RCH_2CH{=}CH\overset{O}{\overset{\|}{C}}SCoA + TPNH + H^+ \rightleftharpoons RCH_2CH_2CH_2\overset{O}{\overset{\|}{C}}SCoA + TPN^+ \quad (4)$$

Reaction 1 and 2 occur in rapid sequence with reaction 2 yielding the driving force for net condensation by removal of the postulated keto acid intermediate. Reaction 3 is a dehydration reaction. It is not known whether the same enzyme can yield the Δ^2 and Δ^3 acids or whether the Δ^2 and Δ^3 may be directly interconvertible by action of an isomerase. Reaction 4 is the reduction of the Δ^2 acid with TPNH being the redox cofactor. Again, it is not known if the Δ^3 acid can also be reduced.

This reaction sequence is different from that described by Nugteren (1965) for the microsomal chain elongation system. In the microsomal system, malonyl-CoA is the condensing unit and TPNH is the only reductant necessary. There was no evidence that a Δ^3 acid could be formed by the microsomal enzymes.

Why the liver has two chain elongation systems located in two different compartments of the cell, the mitochondria and the microsomes (presumably the endoplasmic reticulum), is not known. Nor is the quantitative role of either in the economy of fatty acid metabolism known. It is tempting to speculate, since most of the synthesis *de novo* seems to occur in the cytoplasm of the cell, that the endoplasmic reticulum chain elongation system is perhaps more accessible and is thus more involved with producing fatty acids of chain length greater than 16:0. The chain elongation system in the mitochondria might have a lesser role in total net yield of long-chain acids of the organism and is more involved in the fatty acid economy of the mitochondria itself.

References

Ahrens, H. A., Jr., Hirsch, J., Peterson, M. L., Insull, W., Jr., Stoffel, W., and Farguhas, J. W. (1959), *Lancet 1*, 115.

Barron, E. J. (1966), *Biochim. Biophys. Acta 116*, 425.

Barron, E. J., and Hanahan, D. J. (1958), *J. Biol. Chem. 231*, 493.

Barron, E. J., and Mooney, L. A. (1968), *Anal. Chem. 40*, 1742.

Borgstrom, C. (1952), *Acta Physiol. Scand. 25*, 101.

Davidoff, F., and Korn, E. D. (1964), *J. Biol. Chem. 239*, 2496.

Erwin, J., and Bloch, K. (1964), *Science 143*, 1006.

Freedman, R. W., and Becker, E. J. (1951), *J. Amer. Chem. Soc. 73*, 2366.

Goldman, P., and Vagelos, R. P. (1961), *J. Biol. Chem. 236*, 2620.

Harlan, W. R., and Wakil, S. J. (1962), *Biochem. Biophys. Res. Commun. 8*, 131.

Harlan, W. R., and Wakil, S. J. (1963), *J. Biol. Chem. 238*, 3216.

Howton, D. R., and Davis, R. H. (1951), *J. Org. Chem. 16*, 1405.

Jenny, E. F., and Grob, C. A. (1953), *Helv. Chim. Acta 36*, 1936.

Kass, L. R. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1967, 243 C.

Lennarz, W. J., Light, R. J., and Bloch, K. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 840.

Lynen, F. (1961), *Fed. Proc. 20*, 941.

Metcalf, L. D., and Schmitz, A. A. (1961), *Anal. Chem. 33*, 363.

Mooney, L. A., and Barron, E. J. (1970), *Biochemistry 9*, 2138.

Nugteren, D. H. (1965), *Biochim. Biophys. Acta 106*, 280.

Porter, J. W., and Long, R. W. (1958), *J. Biol. Chem. 223*, 20.

Scheuerbrandt, G., Goldfine, H., Baronowsky, P. E., and Bloch, K. (1961), *J. Biol. Chem. 236*, PC 70.
Schlenk, H., and Gellerman, J. L. (1960), *Anal. Chem. 32*, 1412.
Shriner, R. L. (1942), *in* Organic Reactions, Coll. Vol. 1, Adams, R., Ed., New York, N. Y., Wiley, p 1.
Stallberg-Stenhagen, S., and Stenhagen, E. (1944), *Ark. Kemi Mineral. Geol. 19A*, 1.
Tulloch, A. (1964), *J. Amer. Oil Chem. Soc. 41*, 833.
Vandenheuvel, F. A. (1956), *Anal. Chem. 28*, 362.
Von Rudloff, E. (1956), *Can. J. Chem. 34*, 1413.
Wieland, O., and Rueff, L. (1953), *Angew. Chem. 184*, 186.

Phosphonic Acid Metabolism in *Tetrahymena**

Joseph Donald Smith† and John H. Law‡

ABSTRACT: The uptake and metabolism of 2-[^{14}C]-(β-phosphono)alanine and 1,2-[^{14}C]aminoethylphosphonic acid by *Tetrahymena pyriformis* were studied *in vivo*. The aminoethylphosphonic acid was taken up and incorporated into phospholipids to the extent of 10% without any degradation. About 19% of the (β-phosphono)alanine taken up by the cell during growth was found as aminoethylphosphonic acid in the phospholipids.

No (β-phosphono)alanine was detected at the phospholipid level. Some of the radioactive label from (β-phosphono)alanine was also found in the fatty acids and glycerol ethers.

In the past few years the phospholipids of *Tetrahymena pyriformis* have been the center of intensive interest because of the presence of an unusual phospholipid base, 2-aminoethylphosphonic acid (Kandatsu and Horiguchi, 1962). In the phospholipids AEP[1] occurs linked to ceramide (Carter and Gaver, 1967) and to diglyceride and 2-acylchimyl alcohol (Rosenberg, 1964; Thompson, 1967). AEP is also present in the free form and covalently linked to an insoluble residue (Rosenberg, 1964).

In addition to AEP, another phosphonic acid, (β-phosphono)alanine, is found in *Tetrahymena* (Kittredge and Hughes, 1964). While absent from the lipids of *Tetrahymena*, it is present in the free form and in the insoluble residue. PAla is presumed to be a biosynthetic precursor of AEP (Kittredge and Roberts, 1969). The literature on biosynthesis of the carbon–phosphorus bond has been recently reviewed (Kittredge and Roberts, 1969).

Liang and Rosenberg (1966) showed that homogenates of *Tetrahymena* could incorporate ^{32}P-labeled AEP into phospholipids. Warren (1968) reported a small conversion (0.04%) of ^{32}P-labeled PAla to AEP by extracts of *Tetrahymena*. While these authors have reported the ability of *Tetrahymena* to perform these reactions, there has been no report of the actual capacity of the organism to metabolize AEP and PAla, *in vivo*.

In the work presented here we have studied the uptake of 1,2-[^{14}C]AEP and 2[^{14}C]PAla by *Tetrahymena* during its growth and their metabolism by the organism.

Materials

2-[^{14}C]Sodium pyruvate (10 μCi/μmole) was obtained from International Chemical and Nuclear Corp., and 1,2-[^{14}C]-ethanolamine-HCl (117 μCi/μmole) from Tracerlab. Pyruvic acid was purchased from Calbiochem and ethanolamine from Aldrich Chemical Co.

Dimyristoylglyceryl-AEP (PnE) was the gift of Dr. Erich Baer.

The sources of other materials have been previously reported (Smith and Law, 1970).

Methods

Synthesis of 2-[^{14}C]Phosphonoalanine. 2-[^{14}C]Sodium pyruvate (500 μCi) to which 750 μmoles of nonradioactive pyruvic acid had been added was used for the synthesis of 2-[^{14}C]-PAla. The intermediate *N*-acetyldehydroalanine was prepared by the method of Wieland *et al.* (1957), and PAla by the procedure of Chambers and Isbell (1964). To pyruvate was added 73.4 mg of acetamide and 5 ml of benzene. The mixture was heated under reflux for 5 hr with a trap containing $CaSO_4$ to remove the water formed in the reaction. The benzene was then evaporated under nitrogen and a fivefold molar excess of each of trimethyl phosphite and dimethyl phosphite was added. After heating on a steam bath for 90 min, the reaction mixture was left at room temperature. After

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[1] Abbreviations used are: AEP, 2-aminoethylphosphonic acid; PAla, (β-phosphono)alanine; PnE, diacylglyceryl-AEP; PE, phosphatidylethanolamine.