groups on the nitrogen atom of the ring-opened forms increase the rate of pseudo base formation in the ring-closure reaction.

The substituent effects of both the third-order ring-opening reaction and the overall ring-opening equilibrium constant are the cumulative result of two separate reactions with the unstable pseudo base as the intermediate. Because the substituent effects are in the same direction for these two reactions the total substituent effect is large.

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In Vitro Synthesis of Lignoceric and Nervonic Acids in Mammalian Liver and Brain*

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ABSTRACT: A fatty acid elongation system has been identified in 21-day-old rat liver and brain mitochondria. This fatty acid system will elongate a multitude of saturated and unsaturated acyl coenzymes (acyl-CoAs) ranging in chain length from C_{12} to C_{22}. The components necessary for this elongation have been found to be a precursor acyl-CoA, acetyl coenzyme A reduced nicotinamide–adenine dinucleotide and reduced nicotinamide–adenine dinucleotide phosphate. Acetyl-CoA and not malonyl-CoA has been found to be the immediate precursor of the two-carbon addition unit. It was this mitochondrial system which was found to elongate behenyl-CoA to lignoceric acid and erucyl-CoA to nervonic acid. The synthesized radioactive lignoceric and nervonic acids were identified by thin-layer and gas chromotography. Their response to hydrogenation was further proof of their identity. Decarboxylation of the [^{14}C]ligonceric and nervonic acids revealed they had both been synthesized by an elongation process.

Studies *in vivo* have shown that the brain is capable of synthesizing lignoceric ($C_{24:0}$) and nervonic acids ($C_{24:1}$ Δ^{15}). In these studies radioactive lignoceric and nervonic acids were isolated from the brains of animals which had previously been injected with [1-^{14}C]acetate. These acids were then decarboxylated in an effort to determine the mechanism by which they had been synthesized. Fulco and Mead (1961) found an even distribution of radioactivity in the isolated lignoceric acid and therefore postulated that it has been synthesized by a *de nono* process. Bernhard *et al.* (1962), Hajra and Radin (1963a,b) and Kishimoto and Radin (1963a) felt however that lignoceric acid had been synthesized by an elongation process because they observed a preferential location of radioactivity at the carboxyl end of the fatty acid. Fulco and Mead (1961), Hajra and Radin (1963b), and Kishimoto and Radin (1963b) agreed that the decarboxylation data indicated an elongation process was responsible for the synthesis of nervonic acid.

Evidence will be presented in this paper that the enzyme system used for elongation is intramitochondrial. The components necessary for fatty acyl elongation will be documented using a system which elongates palmityl-CoA to stearic acid.

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Evidence will then be presented supporting acetyl-CoA as the immediate precursor of the two-carbon elongation unit. Finally using the mitochondrial elongation system *in vitro* with all components being optimal it will be shown that behenyl-CoA ($C_{22:0}$ CoA) and erucyl-CoA ($C_{22:1}$ Δ^{13}-CoA) are elongated to lignoceric and nervonic acids, respectively.

Methods and Materials

Preparation of Mitochondria. Rat brain and liver tissues were obtained from 21-day-old albino rats which had been weaned on the same day. Eight to ten rats were decapitated and the brains or livers were removed intact. The tissues were first washed in cold 0.25 M sucrose (pH 7.2) and then homogenized for 2 min in the cold sucrose (9 ml/g of tissue) with a Potter-Elvehjem-type homogenizer fitted with a Teflon pestle. The nuclear and mitochondrial fractions were prepared according to the procedure of Schneider (1948), as modified by Lardy and Wellman (1952). The twice-washed mitochondrial pellets were then suspended with 3 ml of 0.25 M sucrose/g of original tissue. This mitochondrial suspension was then sonicated for 30 min at setting of 8–9 A on a Branson's Instrument Sonifier. The sonification was done in 5-min intervals to prevent overheating. At no time during the sonification did the temperature rise above 10°. The sonicated suspension was then spun at 100,000*g* for 1 hr. The residue was suspended in 0.25 M sucrose (0.2 ml used per g of original tissue).

Protein in all fractions was determined by the Biuret method (Gornall *et al.*, 1949).

Assay of Mitochondrial Purity. Succinic dehydrogenase (as a characteristic mitochondrial enzyme was measured manometrically according to the method of Slater and Bonner (1952). Microsomal contamination of the mitochondrial pellet was estimated by measuring the amount of glucose 6-phosphatase (Swanson, 1955) and by electron microscopy. The mitochondrial pellets were fixed in 1% OsO_4 which had been buffered to pH 7.4 by Veronal–acetate buffer (Palade, 1952). Slices were then dehydrated and embedded in British araldite. Electron micrographs were taken on Kodak Lantern Slide plates using a Philips 100B electron microscope.

Preparation of Substrates. Acetyl-CoA was prepared chemically by the thiophenol method (Wieland and Köppe, 1953), while [1-^{14}C]acetyl-CoA was prepared enzymatically by using the acetate-activating enzyme (Hele, 1954). Malonyl-CoA and radioactive malonyl-CoA were synthesized as previously described (Bressler and Wakil, 1961). Fatty acyl-CoA derivatives were prepared enzymatically by the fatty acid activating enzyme found in guinea pig microsomes (Kornberg and Pricer, 1953). The fatty acids obtained by hydrolysis from the synthesized CoAs were analyzed by gas chromatography and found to contain less than 2% impurity. The following substrates and cofactors were obtained from commercial sources: ATP, NADPH, NADH, avidin, and glucose 6-phosphate.

Assay of Fatty Acid Synthesis. The elongation of fatty acids was assayed by determining the amount of [^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA incorporated into the longer chain fatty acids. Routinely the assay mixture contained 1.0 mg of protein, 4–80 mμmoles of [1-^{14}C]acetyl-CoA (2×10^3 cpm/mμmole) or 30–65 mμmoles of [1,3-^{14}C]malonyl-CoA (2×10^3 cpm/mμmole) where indicated, 1 μmole of NADH, 1 μmole of NADPH, 40 mμmoles of acyl-CoA, and 40 μmoles of potassium phosphate buffer (pH 7.2). The total volume was made up to 0.5 ml by the addition of H_2O. The *de novo* synthesis of fatty acids was measured by the amount of [^{14}C]malonyl-CoA incorporated into palmitic acid. The reaction mixture for the assay of this synthesis contained 1 mg of protein, 30–65 mμmoles of [1,3-^{14}C]malonyl-CoA (2×10^3 cpm/mμmole), 1 mμmole of acetyl-CoA, 1 μmole of NADPH, and 40 μmoles of potassium phosphate (pH 6.5). The total volume was adjusted to 0.5 ml by the addition of H_2O. All reaction mixtures were flushed with nitrogen, capped, and incubated from 10 to 60 min at 38°. The reactions were stopped by the addition of 0.2 ml of a 10% alcoholic potassium hydroxide solution. The complete reaction mixture was then saponified for 20 min in a boiling-water bath followed by acidification with 2.0 ml of 4 N HCl. The acidified mixture was extracted three times with pentane. Under these extracting conditions [^{14}C]acetic acid [^{14}C]malonic acids are not extracted with pentane. The pentane extracts were then collected, the solvent was evaporated to dryness, and the residue was methylated with diazomethane. An aliquot of the methyl esters was pipetted into a counting vial containing toluene and a scintillation mixture of 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene. The radioactivity was then measured in a Packard Tri-Carb liquid scintillation spectrometer.

Identification of the [^{14}C]Fatty Acids. Identification of the fatty acid methyl esters was accomplished by a combination of various analytical procedures that included gas–liquid partition and thin-layer chromatography. A Barber-Coleman Model 10 gas–liquid partition chromatograph was used with a radium ionization detector. Glass columns either 6 or 8 ft in length were packed with 15% ethylene glycol succinate on Chromosorb W (60–80 mesh) and the column was operated at 165–175° with argon gas (flow rates of 100–125 ml/min). The identification of the unknown radioactive methyl esters was accomplished by comparing their retention times to those of known standard methyl esters which were included in each aliquot that was chromatographed.

The radioactive methyl esters in the effluent gas were trapped in glass capsules 5 cm in length containing Pyrex wool (Harlan and Wakil, 1963). A Packard gas chromatograph fraction collector was used to facilitate these collections. The capsules were placed in scintillation vials, 20 ml of scintillation mixture was injected into the vial through the capsule, and the vial and its contents were counted. Between 80 and 90% of the radioactivity injected into the column was recovered with this method of collection. For further analysis individual fatty acid fractions were collected in the same manner and eluted from the Pyrex wool with pentane.

Individual fatty acid fractions collected from the gas chromatograph were treated in two ways to determine whether they were saturated or unsaturated. An aliquot from the collected fraction was hydrogenated along with a sample of standard nonradioactive methyl oleate and then rechromatographed (Farquhar *et al.*, 1959). If the treated fatty acid methyl ester had the same retention time as it had before hydrogenation, it was assumed to be a saturated fatty acid of the same chain length as the standard methyl ester with which it had been collected originally. The shortening of the retention time of the standard methyl oleate to that of methyl stearate indicated the completion of the hydrogenation pro-

cess. Aliquots of the [^{14}C]fatty acids were also chromatographed by thin layer on a silica plate impregnated with $AgNO_3$ (10%), using ether–hexane (1:9) as the developing solvent (Morris, 1962). In this system the methyl esters of the saturated, mono-, di-, and polyunsaturated fatty acids are separated from each other. Standard methyl esters were run on the same plate. The position of the various esters was located by spraying with a 2% alcoholic solution of 2′,7′-dichlorofluorescein and viewing the plate under an ultraviolet lamp. The distribution of the radioactive methyl esters was determined by scraping off 1-cm segments from the plate beginning 0.5 cm below the origin and continuing to the front. The scrapings from each centimeter segment were placed in counting vials containing the toluene scintillation mixture and counted in the Packard Tri-Carb liquid scintillation spectrometer.

To verify the carbon chain length of the various fatty acid esters an aliquot of the sample collected from the initial gas chromatograph was rechromatographed on a reverse-phase, silica gel G plate with acetonitrile–acetic acid–water (70:10:25) as the developing solvent (Mangold, 1961). The location of the radioactive fatty acid methyl ester was determined as described earlier. In this system the shorter chain esters moved closer to the front thus allowing a separation and identification of different chain length fatty acid methyl esters. The radioactive peaks were identified by comparing their R_F's to those of standard fatty acid methyl esters which had been run on the same plate.

The location of the double bond in nervonic acid was accomplished by a microoxidation procedure using a permanganate–periodate mixture (von Rudloff, 1956; Scheuerbrandt and Bloch, 1962). The isolated radioactive methyl ester which had a retention time identical with standard methyl nervonate was subjected to this analytical oxidation procedure. It is to be remembered that the aliquot of tentatively identified radioactive methyl ester contained some standard nonradioactive methyl nervonate (used as an internal standard). Nonradioactive methyl oleate was added to the aliquot to be oxidized in order to prevent a nonspecific association of the radioactive oxidation products with the oxidation products of the nonradioactive standard methyl nervonate. Methyl esters of the oxidation products were then prepared by adding freshly generated diazomethane. The dicarboxylic acid methyl esters were then identified using the same gas chromatographic conditions as stated. At this temperature setting the monocarboxylic methyl esters were not identifiable.

Decarboxylation of [^{14}C]*Fatty Acids.* The methyl esters which had been isolated by gas chromatography were saponified with 10% alcoholic potassium hydroxide in a boiling water bath for 20 min. The fatty acids were isolated and then decarboxylated *via* the Schmidt reaction according to the procedure of Brady (1960).

Avidin and Biotin Assays. Biotin was measured microbiologically by the method of Snell and Wright (1941). Avidin was assayed by two different methods. Avidin was first assayed according to a method of Greene (1963). This method measured the ability of avidin to bind [^{14}C]biotin. Avidin was also measured (Halenz and Lane, 1960) by its ability to inhibit propionyl carboxylase (a biotin-containing enzyme), which was found to be present in the residue isolated from sonicated mitochondria.

Propionyl-CoA Carboxylase Assay. Propionyl carboxylase was measured according to the method of Halenz and Lane (1960).

Acetyl-CoA Carboxylase Assay. Acetyl-CoA carboxylase activity was determined by measuring the amount of [^{14}C]-bicarbonate incorporated into malonyl-CoA (Waite and Wakil, 1962).

Malonyl Decarboxylase Assay. Malonyl decarboxylase activity was determined by incubating [1,3-^{14}C]malonyl-CoA with the enzymatic fraction in question and measuring the amount of radioactive CO_2 trapped in Hyamine in a closed container. The [1,3-^{14}C]malonyl-CoA was injected through a rubber stopper into the closed container containing the reaction components which was then incubated in a shaker bath for 1 hr at 38°. The containers were then placed in ice for 5 min and unstoppered. The vial containing the radioactive CO_2-trapped Hyamine was removed and counted in the toluene scintillation mixture. The [^{14}C]fatty acids were then extracted from the reaction mixture as previously described. The amount of fatty acid synthesis was determined to illustrate that the radioactive CO_2 produced was not dependent on the amount of fatty acid synthesis.

Results

Homogeneity of the Mitochondrial Fraction. A high concentration of succinic dehydrogenase (14.4 μl of O_2/hr per mg) was found in a sample of intact mitochondria. The level of activity (24.8 μl of O_2/hr per mg) was increased in the sonicated mitochondrial residue of the same sample. In this same sample no glucose 6-phosphatase activity was found in the intact or sonicated mitochondrial residue. The level of activity in microsomes from the same preparation was 5.0 units/mg. Morphologically electron micrographs revealed a minimal microsomal contamination of the mitochondrial pellet.

Assay of Fatty Acid Synthesizing Systems in Mitochondria. A summary of the results obtained in a typical experiment from assaying the mitochondrial fractions for both *de novo* and elongation activity is presented in Table I. Elongation system 1 used [1-^{14}C]acetyl-CoA as the C2 donor while elongation system 2 used [1,3-^{14}C]malonyl-CoA as the C2 donor. A greater amount of synthesis was obtained when [1-^{14}C]-acetyl-CoA was used. The residue isolated from sonicated mitochondria was found to have the highest fatty acid elongation activity (18.9 mμmoles of [^{14}C]acetyl-CoA incorporated/hr per mg of protein). Intact mitochondria had a much lower activity of 2.2 suggesting that the elongation system is intramitochondrial. The very low activity of 0.8 in the sonicated mitochondrial supernate suggests that the enzyme system is firmly attached to the inner membrane structure. There was practically no *de novo* synthesis in any of the mitochondrial fractions.

The [^{14}C]fatty acids obtained from the reactions which resulted in a significant amount of fatty acid synthesis were isolated, identified, and decarboxylated in order to verify their pathway of synthesis. Stearic acid should be synthesized by the elongation process while palmitic acid should be synthesized by the *de novo* process. Upon decarboxylation 100% of radioactivity of stearic acid should be found in the carboxyl group. Each odd-numbered carbon of palmitic acid should be radioactive if it was synthesized by the *de novo* process from [1,3-^{14}C]malonyl-CoA. With this even distribu-

TABLE I: Mitochondrial Location of Fatty Acid Synthesis.[a]

elongation system 1 = [^{14}C]acetyl-CoA + palmityl-CoA ⟶ [^{14}C]stearyl-CoA
elongation system 2 = [^{14}C]malonyl-CoA + palmityl-CoA ⟶ [^{14}C]stearyl-CoA
de novo system = [^{14}C]malonyl-CoA + acetyl-CoA ⟶ [^{14}C]palmitic acid

Fraction	Elongation System 1	Elongation System 2	*De Novo* System
Intact mitochondria	2.2	0.5	0.06
Sonicated mitochondrial residue	18.9	4.4	0.09
Sonicated mitochondrial supernatant (S_1)	0.8	0.2	0.06

[a] The following systems were used. Elongation system 1 contained 50 mμmoles of [1-^{14}C]acetyl-CoA (6×10^2 cpm/mμmole), 40 mμmoles of palmityl-CoA, 1 μmole of NADH, 1 μmole of NADPH, and 40 μmoles of potassium phosphate buffer (pH 7.2). Elongation system 2 contained 30 mμmoles of [1,3-^{14}C]malonyl-CoA (5×10^3 cpm/mμmole), 1 mμmole of acetyl-CoA, 40 mμmoles of palmityl-CoA, 1 μmole of NADH, 1 μmole of NADPH, and 40 μmoles of potassium phosphate buffer (pH 7.2). The *de novo* system contained 30 mμmoles of [1,3-^{14}C]malonyl-CoA (5×10^3 cpm/mμmole), 1 mμmole of acetyl-CoA, 1 μmole of NADPH, and 40 μmoles of potassium phosphate buffer (pH 6.5). All tubes were made up to a total volume of 0.5 ml with distilled water and incubated 1 hr at 38°, and the results were expressed as millimicromoles of radioactive precursor incorporated per hour per milligram of protein.

tion of radioactivity throughout the molecule only 12.5% of the total radioactivity should be found in the carboxyl group.

[^{14}C]Stearic acid was found to be the main product when the residue from sonicated mitochondria was mixed with the elongation assay containing palmityl-CoA and either [^{14}C]-acetyl-CoA or [^{14}C]malonyl-CoA. The stearic acid was identified and isolated by gas chromatography. When the stearic acid was decarboxylated 90% of the total radioactivity was found in the end carboxyl group. A greater amount of [^{14}C]acetyl-CoA was incorporated into stearic acid than was [^{14}C]malonyl-CoA, thus suggesting that acetyl-CoA was the direct precursor of the active two-carbon unit used in elongation.

Components Necessary for the Elongation Process. The components necessary for the elongation of palmityl-CoA to stearyl-CoA are shown in Table II. The complete system contains enzyme, [1-^{14}C]acetyl-CoA, palmityl-CoA, NADH, NADPH, and phosphate buffer. There was no synthesis when palmityl-CoA was omitted, nor was there any synthesis when both NADH and NADPH were omitted. The omission of either nucleotide seems to depress elongation process about 60%.

TABLE II: Components Necessary for the Elongation of Palmityl-CoA to Stearyl-CoA.[a]

Additions and Omissions	mμmoles of [^{14}C]-Acetyl-CoA Incorpd into Stearic Acid
None	5.20
Less palmityl-CoA	0.26
Less NADH	1.55
Less NADPH	1.49
Less NADH + NADPH	0.02

[a] The complete system contained 41 mμmoles of [1-^{14}C]-acetyl-CoA (2×10^3 cpm/mμmole), 40 mμmoles of palmityl-CoA, 1.5 μmoles of NADH, 1.5 μmoles of NADPH, 40 mμmoles of potassium phosphate buffer 1.0 mg of residue from sonicated liver mitochondria, and water to a total volume of 0.5 ml. The mixture was incubated for 10 min.

Effect of Time of Sonication of the Mitochondria on the Elongation System. The residue isolated from sonicated mitochondria had a much greater specific activity for elongation than did the intact mitochondria. It was found that the specific activity of the residue from sonicated mitochondria isolated by spinning at 100,000*g* for 1 hr increased the longer the mitochondrial suspension was sonicated. Maximal specific activity was reached at 30-min sonication. Sonication for 1, 2, or 3 hr did not increase the specific activity. As stated before sonication was done in 5-min intervals to prevent overheating.

Effect of Incubation Time on the Elongation of Fatty Acids. The elongation reaction was measured at various time intervals by determining the amount of [^{14}C]acetyl-CoA incorporated into stearic acid when palmityl-CoA was the cosubstrate. The amount of [^{14}C]acetyl-CoA incorporated appeared to be linear for the first 30-min incubation.

Effect of Amount of Enzyme on the Elongation of Fatty Acids. When increasing amounts of enzyme protein were used in the incubation mixtures it was seen that the amount of [^{14}C]acetyl-CoA incorporation was linear between 0.5 and 1.5 mg of protein (Figure 1). The incubations were stopped after 10 min while the time response was linear. The nonlinear synthesis at low protein concentrations may be characteristic of a multienzyme system where one or more enzyme(s) may be present in limited amounts.

Effect of pH on the Elongation of Fatty Acids. The pH optimum was found to be between 7.0 and 8.0. This conclusion was reached after measuring the [^{14}C]acetyl-CoA incorporation in reaction mixtures containing phosphate buffers of varying pH. The pH was varied from 5.8 to 8.0 at intervals of 0.2 pH unit as shown in Figure 2.

Effect of NADH and NADPH on the Elongation of Fatty Acids. The effect of concentrations of both NADH and NADPH on activity is illustrated in Figures 3 and 4. The optimal level of each nucleotide was found to be about 1.5 μmoles. The effect of increasing the amount of one of the nucleotides was studied both in the absence of the other nucleotide or with a constant amount (1 μmole) of the other nucleotide. In the absence of one of the nucleotides the

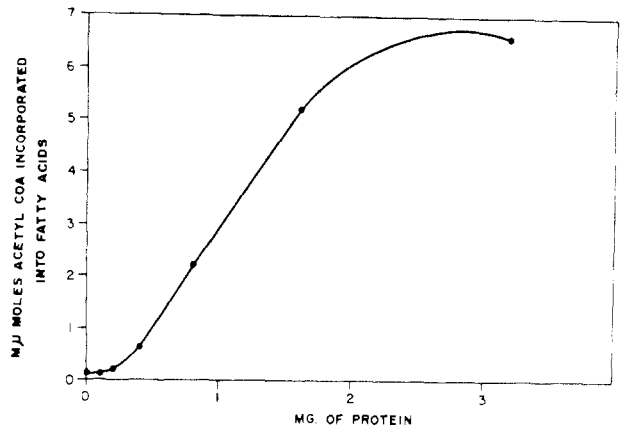

FIGURE 1: Effect of protein on the elongation of fatty acids. The reaction mixture contained 41 mμmoles of [1-^{14}C]acetyl-CoA (2×10^3 cpm/mμmole), 40 mμmoles of palmityl-CoA, 1 μmole of NADH, 1 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), and increasing amounts of protein from the residue of sonicated mitochondria. Final volume was 0.5 ml and the mixture was incubated for 10 min.

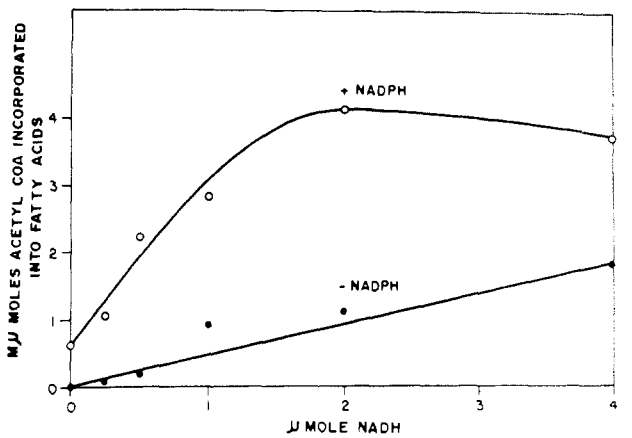

FIGURE 3: Effect of concentration of NADPH on the elongation of fatty acids. The same reaction mixture reported in Figure 1 was used except that 1 mg of protein was added and increasing amounts of NADPH were added in the presence of either 0 or 1.0 μmole of NADH. The mixture was incubated for 10 min.

activity of the elongation process is markedly depressed and increasing the amounts of the one nucleotide causes very little increase in activity. At relatively high concentration of NADH there was a significant amount of elongation in the absence of NADPH suggesting that NADH may partially substitute for NADPH. The requirement for both reduced pyridine nucleotides in the liver and brain mitochondria seem to be different than the heart mitochondrial system in which only NADH was required (Dahlen and Porter, 1968).

Effect of [1-^{14}C]Acetyl-CoA Concentration on the Elongation of Fatty Acids. When 40 mμmoles of palmityl-CoA was used in each incubation tube a maximum rate of elongation was reached after about 70 mμmoles of [^{14}C]acetyl-CoA had been added. Further increases in [^{14}C]acetyl-CoA concentration up to 200 mμmoles did not cause any significant changes in rate. The K_m for acetyl-CoA was found to be 7.14×10^{-6} M.

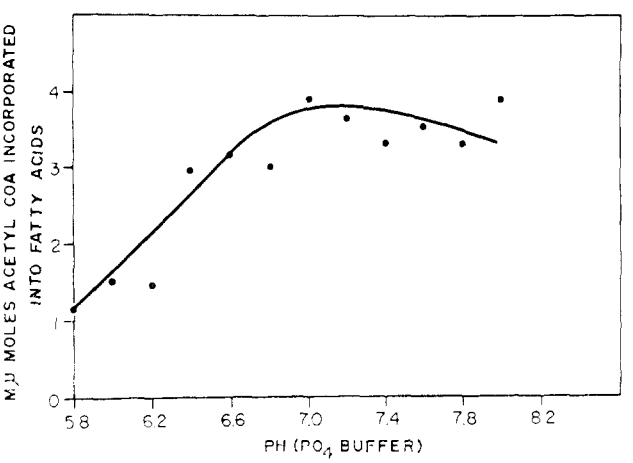

FIGURE 2: Effect of pH on the elongation of fatty acids. The same reaction mixture reported in Figure 1 was used except that 1 mg of protein was added and the pH of the phosphate buffer was varied. The incubation time was 10 min.

Elongation of Various Chain Length Acyl-CoAs. It was of importance to determine the specificity of elongation enzymes for various acyl-CoAs because if the range of specificity was wide it would be possible to postulate that fatty acids having chain lengths greater than palmitic acid were probably synthesized by the elongation process. Therefore elongation of the following saturated acyl-CoA was attempted; $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{20:0}$, and $C_{22:0}$. The monounsaturated acyl-CoAs ($C_{16:1}$ Δ^7 and $C_{18:1}$ Δ^9) were also tested. These results are shown in Figure 5. It was found that all the acyl-CoAs tested were capable of being elongated. The shorter acyl-CoAs were elongated more readily than the longer acyl-CoAs. The monounsaturated acyl-CoAs caused a greater incorporation of radioactivity than did their saturated homologs of the same chain length. Thus it would appear that a greater specificity is shown toward the shorter unsaturated acyl-CoAs either specifically or nonspecifically because of the increased solubilities of such acyl-CoAs.

Malonyl-CoA as an Intermediate in the Elongation Process. Since both malonyl-CoA and acetyl-CoA can be used as a precursor in the elongation of acyl-CoAs, it is of major importance to determine which of these is the direct precursor of the active two-carbon unit. Since radioactivity from both of these CoAs is capable of being incorporated in the elongation process, there is probably a conversion from one into another which takes place by either mechanism 1 or 2.

$$\text{acetyl-CoA} + CO_2 \xrightarrow[\text{carboxylase}]{\text{acetyl-CoA}} \text{malonyl-CoA} \qquad (1)$$

$$\text{malonyl-CoA} \xrightarrow[\text{decarboxylase}]{\text{malonyl-CoA}} CO_2 + \text{acetyl-CoA} \qquad (2)$$

It is also possible that the conversion could be the result of the reversal of one of the above equations. The experimental data to be presented indicate that the sequence seen in eq 2 is the one in operation in mitochondria. As seen in Table I, the rate of acetyl-CoA incorporation was better than that of malonyl-CoA thus suggesting that acyl-CoA might be the

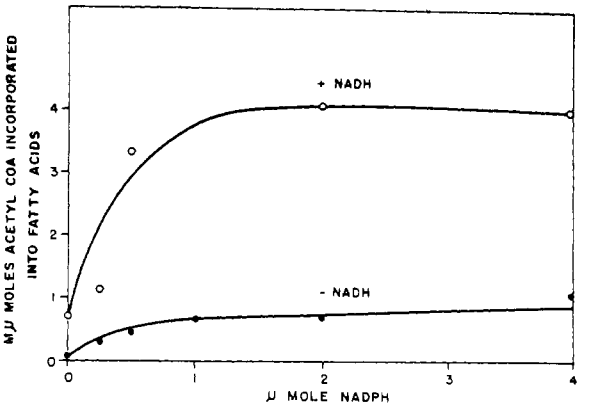

FIGURE 4: Effect of concentration of NADH on the elongation of fatty acids. The same reaction mixture reported in Figure 1 was used except that 1 mg of protein was added and increasing amounts of NADH were added in the presence of either 0 or 1.0 μmole of NADPH. The mixture was incubated for 10 min.

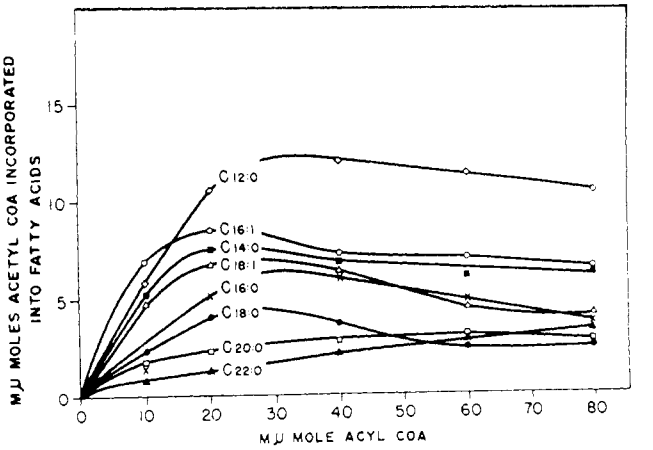

FIGURE 5: Elongation of increasing chain-length fatty acyl-CoAs. The reaction mixture contained 82 mμmoles of [1-^{14}C]acetyl-CoA (3×10^3 cpm/mμmole), varying amounts of acyl-CoAs, 1.5 μmoles of NADH, 1.5 μmoles of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), 1.0 mg of protein from the residue of sonicated liver mitochondria, and water to a final volume of 0.5 ml. The mixture was incubated for 10 min.

direct precursor of the two-carbon unit used in the elongation process. The following radioactive dilution studies also indicated acetyl-CoA as the direct precursor.

When increasing amounts of nonradioactive acetyl-CoA were added to incubation mixtures containing a constant amount of [1,3-^{14}C]malonyl-CoA, the dilution of radioactivity was found to be very close to the calculated dilution which would be expected if malonyl-CoA was converted into acetyl-CoA before elongation could proceed (Figure 6). Consistent with these results was the finding that there was only a slight dilution of radioactivity when increasing amounts of nonradioactive malonyl-CoA were added to incubation mixtures containing a constant amount of [1-^{14}C]acetyl-CoA (Figure 7). These observations would suggest that malonyl-CoA is converted into acetyl-CoA which is then used in the elongation process.

If malonyl-CoA was the direct precursor of the active two-carbon unit used in elongation, then the reaction sequence as depicted in eq 1 must be operating. Therefore if acetyl-CoA was added to the incubation mixture it would probably be carboxylated to malonyl-CoA by the biotin-containing enzyme, acetyl-CoA carboxylase. Several different assays were used in an effort to determine whether or not acetyl-CoA carboxylase activity was present in the elongation complex of enzymes. A direct assay for the presence of acetyl-CoA carboxylase was attempted by measuring the amount of [^{14}C]HCO_3^- incorporated into malonyl-CoA. Even though there was no incorporation of [^{14}C]HCO_3^- into malonyl-CoA the presence of acetyl-CoA carboxylase could not be ruled out because of the occurrence of a large amount of malonyl decarboxylase activity in the elongation complex of enzymes. It was interesting to note that the residue from sonicated mitochondria contained 57.1 mμmoles of biotin/mg of protein, as assayed microbiologically. The presence of a large amount of propionyl carboxylase (0.75 μmole of HCO_3^- incorporated/hr per mg) (Halenz and Lane, 1960) another known biotin enzyme, accounted for a large proportion of the biotin found by the microbiological assay. However, this finding did not rule out the possible presence of acetyl-CoA carboxylase.

Since the presence of malonyl decarboxylase prevented the direct assay of acetyl-CoA carboxylase it was decided to seek its presence indirectly. Since acetyl-CoA carboxylase was known to be a biotin enzyme it should be specifically inhibited by avidin. If acetyl-CoA carboxylase was necessary for the production of malonyl-CoA before elongation could take place, then avidin should inhibit the elongation process. No such inhibition of elongation was found when avidin was added. Neither citrate which is known to stimulate the acetyl-CoA carboxylase reaction, nor Mn^{2+}, Mg^{2+}, nor HCO_3^- which are components of the carboxylase reaction, had any effect on the amount of fatty acid elongation.

The addition of ATP to the elongation reaction mixture did, however, cause an increased incorporation of [^{14}C]-acetyl-CoA into various long-chain fatty acids. The stimula-

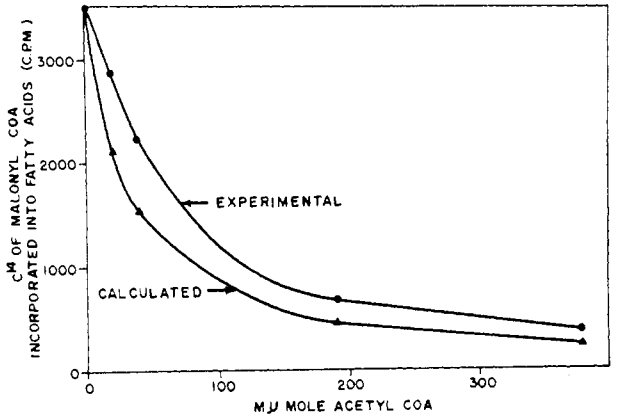

FIGURE 6: Effect of increasing amounts of acetyl-CoA on the incorporation of [1,3-^{14}C]malonyl-CoA. The reaction mixtures contained 30 mμmoles of [1,3-^{14}C]malonyl-CoA (5×10^3 cpm/mμmole), increasing amounts of acetyl-CoA, 40 mμmoles of palmityl-CoA, 1 μmole of NADH, 1 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), 1.0 mg of protein from the residue of sonicated liver mitochondria, and water to a total volume of 0.5 ml. The mixture was incubated for 60 min at 38°.

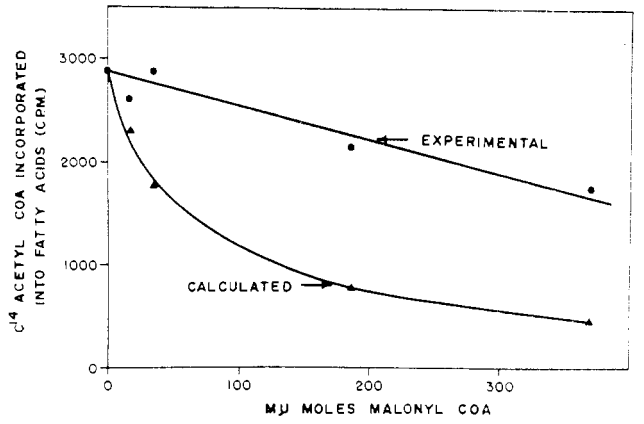

FIGURE 7: Effect of increasing amounts of malonyl-CoA on the incorporation of [^{14}C]acetyl-CoA. The reaction mixtures contained increasing amounts of malonyl-CoA, 70 mμmoles of [1-^{14}C]acetyl-CoA (2.4×10^2 cpm/mμmole), 40 mμmoles of palmityl-CoA, 1 μmole of NADH, 1 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), 1.0 mg of protein from the residue of sonicated liver mitochondria, and water to a total volume of 0.5 ml. The mixtures were incubated for 60 min at 38°.

tion by ATP appears to be due to the activation of the various endogenous fatty acids and not to the carboxylation of acetyl-CoA to malonyl-CoA. This conclusion was reached after studying the elongation of palmityl-CoA, stearyl-CoA, and arachidyl-CoA in the presence and absence of ATP (Table III). When the acyl-CoAs were omitted from the reaction mixture and ATP was added, it was found that some [1-^{14}C]acetyl-CoA was incorporated into long-chain fatty acids. This incorporation was due to the elongation of endogenous fatty acids since more than 50% of the total radioactivity in the synthesized [^{14}C]fatty acids could be released as CO_2 after subjecting the fatty acids to the Schmidt reaction.

TABLE III: Effect of ATP on Elongation.[a]

System	Total cpm	Respective Peaks (cpm) $C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	Other[b]
[1-^{14}C]Acetyl-CoA + ATP	1105	36	289	49	40	691
[1-^{14}C]Acetyl-CoA + $C_{16:0}$ CoA	1404	0	1200	94	0	100
[1-^{14}C]Acetyl-CoA + $C_{16:0}$ CoA + ATP	1950	0	1450	114	0	386
[1-^{14}C]Acetyl-CoA + $C_{18:0}$ CoA	658	6	64	409	24	155
[1-^{14}C]Acetyl-CoA + $C_{18:0}$ CoA + ATP	782	0	84	470	14	214
[1-^{14}C]Acetyl-CoA + $C_{20:0}$ CoA	823	12	44	63	530	174
[1-^{14}C]Acetyl-CoA + $C_{20:0}$ CoA + ATP	983	19	126	42	286	510

[a] The incubation mixtures contained 70 mμmoles of [1-^{14}C]acetyl-CoA (2.4×10^2 cpm/mμmole), 0 or 40 mμmoles of acyl-CoA, 1 μmole of NADH, 1 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), 0 or 8 μmoles of ATP, 1 mg of residue from sonicated liver mitochondria, and water to a total volume of 0.5 ml. The tubes containing the incubation mixtures were flushed with nitrogen, stoppered, and incubated at 38° for 1 hr. Equal amounts were injected into the gas chromatograph. The [^{14}C]fatty acids were collected from the gas chromatograph and counted as was described previously. [b] The radioactivity shown in this column represents the total radioactivity found in fatty acids other than those indicated in other columns. These included various unsaturated fatty acids. No attempt was made to identify these acids.

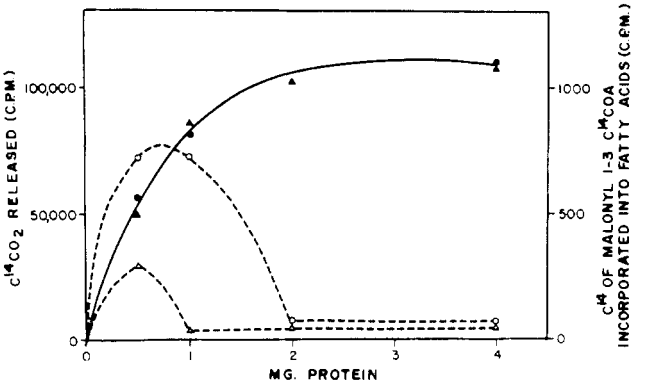

FIGURE 8: Effect of protein on the malonyl-CoA decarboxylase activity. The reaction mixtures contained 60 mμmoles of [1,3-^{14}C]-malonyl-CoA (5×10^3 cpm/mμmole), 40 mμmoles of palmityl-CoA, 0 or 1 μmole of NADH, 0 or 1 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), increasing amounts of protein from the residue of sonicated liver mitochondria, and water to a final volume of 0.5 ml. The solid circles and triangles represent [^{14}C]CO_2 production in the presence and absence of NADH + NADPH, respectively. The open circles and triangles represent fatty acid synthesis in the presence and absence of NADH + NADPH, respectively.

Analysis of the fatty acids synthesized under these conditions revealed the presence of mainly stearic acid and several long-chain unsaturated acids. When ATP was added to the elongation mixture containing palmityl-CoA there was a slight increase of steric and other acids, suggesting that ATP was only activating endogenous fatty acids of which palmitate predominated. Furthermore, ATP did not increase the production of arachidic and behenic acids from stearyl and arachidyl-CoAs, respectively (Table III), but instead there was an incorporation of [^{14}C]acetyl-CoA into stearic acid and several unsaturated fatty acids resulting from the elongation of endogenous acyl-CoAs.

The presence of a large amount of malonyl decarboxylase in the residue from sonicated mitochondria further indicated that malonyl-CoA was not an intermediate in the elongation process. The high rate of CO_2 production as seen in Figure 8 was accredited to malonyl decarboxylase since the small amount of fatty acids synthesis would not explain the large amount of liberated CO_2. The combined omission of NADH and NADPH decreased the amount of fatty acid synthesis but did not effect the amount of CO_2 produced. The presence of malonyl decarboxylase in the same enzyme complex which contained the elongation enzymes explained how

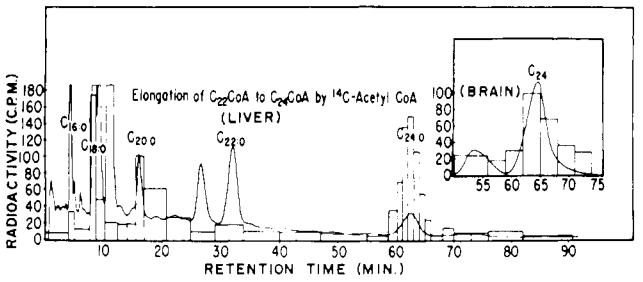

FIGURE 9: Elongation of behenyl-CoA to [^{14}C]lignoceric acid. Gas chromatographic analysis of an aliquot of the methyl esters of fatty acids synthesized from the following incubation mixture: 180 mμmoles of [1-^{14}C]acetyl-CoA (4.6 × 10^3 cpm/mμmole), 40 mμmoles of behenyl-CoA, 1.0 μmole of NADH, 1.0 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), 2.0 mg of protein from the residue of sonicated liver mitochondria (the inset shows results from brain), and water to a final volume of 0.5 ml. The incubation was for 1 hr at 38°.

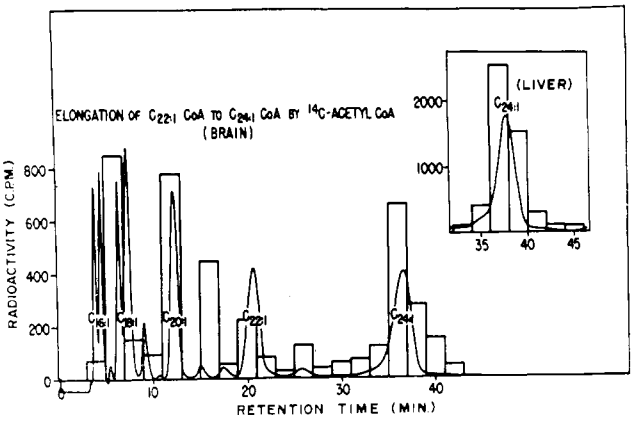

FIGURE 10: Elongation of erucyl-CoA to [^{14}C]nervonic acid. Gas chromatographic analysis of an aliquot of the methyl esters of the fatty acids synthesized from the following incubation mixture: 50 mμmoles of [1-^{14}C]acetyl-CoA (4 × 10^3 cpm/mμmole), 40 mμmoles of erucyl-CoA, 1.0 μmole of NADH, 1.0 μmole of NADPH, 40 μmoles of potassium phosphate buffer (pH 7.2), 1.0 mg of protein from the residue of sonicated brain mitochondria (the inset shows results from liver), and water to a final volume of 0.5 ml.

[1,3-^{14}C]malonyl-CoA could donate the C_2 unit in the elongation process. Therefore it would appear that eq 2 represents the scheme whereby malonyl-CoA was decarboxylated to acetyl-CoA which then served as the immediate precursor of the active two-carbon unit used in the elongation of acyl-CoAs.

The Synthesis of Lignoceric Acid. The components necessary for the synthesis of lignoceric acid were the same as those used in the elongation of palmityl-CoA by [1-^{14}C]acetyl-CoA except that behenyl-CoA was substituted for palmityl-CoA. There was no lignoceric acid synthesized when behenyl-CoA was omitted from the incubation mixture. The omission of either NADH or NADPH decreased the amount of lignoceric produced. When neither of these nucleotides were present there was no lignocerate production. These general properties were very similar to those seen in Table I which illustrates the components necessary for the elongation of palmityl-CoA.

The gas chromatographic analysis of the methyl esters of fatty acids synthesized from [1-^{14}C]acetyl-CoA plus behenyl-CoA is shown in Figure 9. The height of the bars seen in this and the following gas chromatographs represents the amount of radioactivity collected. The width of the bars represents collection time. As seen in Figure 9 a substantial peak of radioactivity was found having the same retention time as that of standard methyl lignocerate. This isolated radioactive methyl ester was identified as [^{14}C]lignoceric acid as follows. This compound was shown to be a saturated acid by two different methods. Following hydrogenation the radioactive methyl ester maintained the same retention time as that of the methyl lignocerate standard. This was indicative of a saturated methyl ester because the retention time of an unsaturated methyl ester would have been decreased by hydrogenation. Thin-layer chromatography on $AgNO_3$-impregnated silica gel plates of this radioactive compound revealed its R_F to be the same as that of a saturated methyl ester. This radioactive methyl ester was shown to be 24 carbons long by gas–liquid partition and thin-layer chromatography. The gas chromatographs seen in Figure 9 indicated that retention time of the radioactive compound was the same as methyl lignocerate ($C_{24:0}$). Reverse-phase thin-layer chromatography also indicated that this unknown methyl ester was 24 carbons long since its R_F was similar to that of the lignocerate standard.

The Synthesis of Nervonic Acid. The components necessary for the synthesis of nervonic acid were found to be the same as those required in the synthesis of lignoceric acid except that erucyl-CoA ($C_{22:1}$ Δ^{13}-CoA) was used instead of behenyl-CoA ($C_{22:0}$ CoA). The gas chromatographic analysis of the fatty acids synthesized from erucyl-CoA plus [1-^{14}C]acetyl-CoA can be seen in Figure 10. There was a large peak of radioactivity whose retention time was the same as that of standard methyl nervonate. When this radioactive methyl ester was chromatographed on $AgNO_3$ thin-layer plates it was observed to have the same R_F as the monoenoic methyl ester standard indicating that the compound was a monoenoic

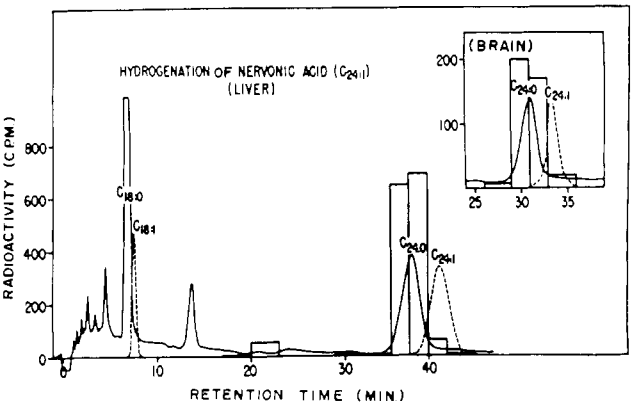

FIGURE 11: Hydrogenation of [^{14}C]nervonic acid. Gas chromatographic analyses of the product methyl esters resulting from the hydrogenation of the nervonic peak collected from the original incubation mixture. In this case the enzyme was from the liver. The inset shows the results from using the brain enzyme. Notice that the large peak with the short retention time is stearic acid, which was produced from the hydrogenation of an added standard methyl oleate.

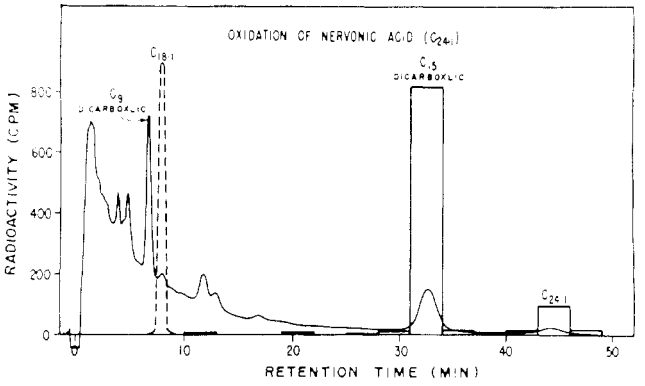

FIGURE 12: Oxidation of [^{14}C]nervonic acid. Gas chromatographic analysis of methyl esters of the dicarboxylic acids which were produced by the permanganate–periodate oxidation of an aliquot of the initially isolated radioactive compound thought to be [^{14}C]nervonic acid. A sample of standard methyl oleate was oxidized in the same reaction mixture.

ester. Hydrogenation of this compound shortened its retention time on gas–liquid partition chromatography from that of a monounsaturated 24-carbon methyl ester to that of a saturated 24-carbon methyl ester (Figure 11).

Reverse-phase thin-layer chromatography also indicated a 24-carbon compound. The radioactive compound was found to have an R_F identical with that of standard methyl nervonate. The location of the double bonds in the C_{15}–C_{16} position was determined after oxidation of the methyl ester with periodate–permangate mixture and gas–liquid partition chromatography of the product. The results showed that the radioactivity was associated with the C_{15}-dicarboxylic methyl ester (Figure 12). These data, therefore, indicated that the [^{14}C]acid obtained from the reaction mixture was nervonic acid.

When [^{14}C]lignoceric and [^{14}C]nervonic acids which had been synthesized from the elongation complex isolated from both liver and brain were decarboxylated, it was found that the majority of the radioactivity was in the carboxyl group (Table IV). Therefore it appeared valid to assume that the [^{14}C]lignoceric and [^{14}C]nervonic had been synthesized by an elongation process.

TABLE IV: Decarboxylation of [^{14}C]Lignoceric and [^{14}C]-Nervonic Acids.

	Liver			Brain		
Acid	Total cpm in [^{14}C]-Fatty Acid	cpm in Carboxyl Group	% ^{14}C in Carboxyl Group	Total cpm in [^{14}C]-Fatty Acid	cpm in Carboxyl Group	% ^{14}C in Carboxyl Group
Lignoceric	548	509	93	128	99	77
Nervonic	500	428	86	151	139	92

Discussion

The activity of the elongation enzyme complex was found to be strictly dependent on the presence of an acyl-CoA (Table II). In the absence of the acyl-CoA there was very little or no incorporation of [1-^{14}C]acetyl-CoA into long-chain fatty acids. Addition of ATP stimulated the elongation of fatty acids due to the activation of endogenous fatty acid to form acyl-CoA. This type of fatty acid activation has been observed in the past (Wakil, 1961; Harlan and Wakil, 1963). The activity of the elongation process was reduced about 60% with the omission of NADH or NADPH. The elongation process was completely inactive if both nucleotides were omitted from the incubation mixture.

Evidence was presented to show that the elongation enzymes were located within the mitochondria. Disruption of the mitochondria by freezing and thawing or by sonication resulted in a 2–10-fold increase in enzymatic activity. The elongation complex appeared to be intimately associated with the intramitochondrial membrane system. The membranal association of the elongation system was so tight that 3-hr sonication failed to solubilize any elongation activity. These findings are very similar to those found by Dahlen and Porter (1968). No attempt was made to determine whether the elongation process was located on the inner or outer membrane of the mitochondria; however our disruption data would indicate that the elongation system was either on the inner membrane or on the inner surface of the outer membrane.

The K_m for acetyl-CoA was found to be 7.14×10^{-6} M. As seen in Figure 5 the shorter acyl-CoAs were elongated at a greater rate than the longer ones. The monounsaturated acyl-CoAs were also elongated faster than their saturated derivatives. The increased activity associated with the shorter unsaturated CoAs may be due to an increased affinity of the enzyme for short unsaturated CoAs or it may be a reflection of the increased solubility of shorter and unsaturated CoAs. Another intriguing possibility is that increasing the chain length of the acyl-CoAs increases their detergent action on the elongating enzymes and hence decreases elongation. Dorsey and Porter (1968) have shown that palmityl coenzyme A seems to inhibit pigeon liver fatty acid synthetase because of its detergent action. The authors found that the *critical mixed micellar concentration* was 5 μM or more, and indeed this is the range used in our experiments. Eger-Neufeldt *et al.* (1965) have shown evidence that increasing the chain length of the acyl-CoA indeed increases the inhibition of enzymatic action. In the gas chromatogram showing the elongation of behenyl-CoA to lignoceric acid and erucyl-CoA to nervonic acids (Figures 9 and 10) a significant amount of radioactivity was found in the respective saturated and monounsaturated shorter chain acids. This may be a reflection of elongation of endogenous shorter chain acyl-CoAs or it may be that the added behenyl- or erucyl-CoAs is partially degraded to shorter acyl-CoAs and then reelongated with acetyl-CoA.

Acetyl-CoA and not malonyl-CoA was found to be the direct precursor of the active two-carbon unit used in the elongation process in mitochondria. This conclusion was based on the following observations: (1) Acetyl-CoA was a better substrate than malonyl-CoA as shown by the faster

rate of its incorporation into the long-chain fatty acids. (2) Nonradioactive acetyl-CoA diluted the amount of [1,3-^{14}C]-malonyl-CoA incorporated into the fatty acids in a proportional amount relative to their concentrations whereas nonradioactive malonyl-CoA did not dilute the amount of [1-^{14}C]acetyl-CoA incorporated into fatty acids. (3) Acetyl carboxylase was not found in the elongation complex by direct assay and avidin did not inhibit the elongation process. (4) A large amount of malonyl decarboxylase activity was found in the same enzyme complex used for elongation. Thus any malonyl-CoA which might be formed would probably have been decarboxylated to acetyl-CoA.

Evidence was presented to show that both lignoceric and nervonic acids are synthesized by an elongation process in both the liver and brain mitochondria. These observations seem to confirm several *in vitro* studies (Fulco and Mead, 1961: Hajra and Radin, 1963a,b).

Microsomes have been found to elongate fatty acids in the presence of ATP using malonyl-CoA as the two-carbon donor (Aeberhard and Menkes, 1968; Nugteren, 1965; Guchhait, 1966). The relative importance of these two elongating systems is not fully appreciated at this time, however it has been shown in this paper that the mitochondrial system is quite active and capable of elongating a multitude of different acyl-CoAs to at least a 24-carbon length. The interplay between the two elongation systems and the palmitate-synthesizing system within mammalian cells is not known at present.

The mechanism of fatty acid elongation in mitochondria is not known. The finding that both NADH and NADPH were necessary for optimal synthesis is not inconsistent with the proposal of Seubert *et al.* (1968) that fatty acid elongation occurs by the reversal of β oxidation of fatty acids. These authors found that combination of purified thiolase, β-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydrase, and enoyl-CoA reductase with acetyl-CoA, NADH, and NADPH resulted in the elongation of acyl-CoA by one or two C_2 units. Although the physiological significance of this system is not known at present, it is possible that this system may be important in the synthesis of mitochondrial fatty acids.

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