Metabolism of 2-Hexadecynoate and Inhibition of Fatty Acid Elongation*

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Dietary methyl-2-hexadecynoate appeared to inhibit fatty acid elongation in intact animals (Wood, R., Lee, T., and Gershon, H. (1980) Lipids 15, 141-150). Data from the present in vitro studies indicate that the microsomal elongation system is inhibited preferentially to the mitochondrial system. A series of metabolic acyl-CoA thioester intermediates has been isolated, characterized, and identified from microsomal and mitochondrial incubations with the 2-hexadecynoic acid (16=1A2). The data support the following conclusions: 1) 16=1A2 is activated to the CoA ester; 2) 16=1A2 is acted on by an isomerase to produce a 2,3-allene; 3) either 16=1A2 or the allene, or both, are hydrated to yield a \( \beta \)-keto-CoA thioester after rearrangement; 4) the \( \beta \)-keto ester is reduced to the \( \beta \)-hydroxyacyl-CoA; 5) dehydrogenation of the \( \beta \)-hydroxy ester gives rise to trans-1A-2-hexadecenoate, which accumulates; and 6) accumulation of the latter results from the inhibition of enoyl-CoA reductase by the 2,3-allene. The occurrence of cis and trans \( \Delta \)-hexadecenoates indicates the allene is reduced, after which the \( \Delta \)-monoenes isomer may be isomerized to the \( \Delta \)-monoenes by the acetylene isomerase or a different enzyme. Indirect evidence suggests that the fatty acid elongation systems may also be inhibited at another site.

The lipids of normal and tumor-bearing (host) rats fed a low level of methyl-2-hexadecynoate were altered dramatically (1). Most liver lipid classes exhibited a massive accumulation of palmitate and palmitoleate with a corresponding decrease in stearate and octadecanoate levels (1). Data from these studies strongly suggested that long chain 2-ynoic acids inhibit elongation of saturated and monoenolic fatty acids. Normal animals were affected to a greater degree than host animals. The present studies are aimed at: 1) determining the effects of 2-ynoic acids on the mitochondrial and microsomal fatty acid elongation systems of normal rat liver; and 2) the identification of the metabolic products of the acetylenic acids.

**Experimental Procedures**

**Materials**—2-Hexadecynoic acid was prepared by the addition of carbon dioxide to the Grignard of 1-pentadecene (2). 3-Hexadecynoic acid was prepared by two methods: treatment of the Grignard of 1-tetradecene with ethylene oxide followed by chromic acid oxidation (2) and the condensation of lithio-1-tetradecene with 2-monobromoacetate according to the procedure of Ames et al. (3). Methyl esters of the acetylenic acids were prepared and purified by preparative TLC on Silica Gel G adsorbent. Both the 2- and 3-ynoic fatty acids were subjected to potassium carbonate isomerization (4), but only the 3-ynoic acid isomerized to yield the 2,3-allene. The 3-ynoate ester also isomerized during GLC. The isomeric mixtures were resolved by HPLC on a octadecyl column (25 cm \times 4.6 mm (internal diameter) using acetonitrile-water (80:20 v/v) solvent system (Fig. 1). The identities of the collected peaks from Fig. 1 were determined by NMR spectroscopy. NMR spectra of methyl-2-hexadecynoate, methyl-3-hexadecynoate, and methyl-2,3-hexadecadienoate were obtained on a Varian XL-200 and are shown in Fig. 2. The chemical shifts for terminal methyl, methylene, and methoxy protons were approximately 0.88, 1.26, and 3.75 ppm, respectively, for all three fatty esters. The methylene hydrogens adjacent to the triple bond of methyl-2-hexadecynoate exhibited a two-proton triplet (J = 7) at 2.33 ppm. The hydrogens at the number two carbon of methyl-3-hexadecynoate gave a two-proton triplet (J = 2.5) at 3.26 ppm, and the hydrogens at carbon number five gave a two-proton multiplet (J = 2.5) at 2.19 ppm. A chemical shift of 2.13 ppm has been reported for the hydrogens at carbon number five of 2,2-dideutero-3-decynoic acid (4). The hydrogens on carbon number five of the allenic ester gave a two-proton quartet (J = 5.5) at 2.11 ppm and the hydrogens at carbon number two and four gave a two-proton multiplet at 5.58 ppm. Some small differences exist between this spectrum and that reported previously for 2,3-decadienonic acid (4).

Partial hydrogenation of the 2- and 3-hexadecynoate esters with the Lindlar catalyst (5) gave predominantly cis-\( \Delta \)-2- and \( \Delta \)-hexadecenoates, respectively, but substantial amounts of the corresponding trans isomers were also produced. Each of the four hexadecienoate isomers were purified by TLC. The relative TLC R\( \text{v} \) values are shown in Fig. 3. The resolution of the geometrical and positional hexadecenoate isomers by capillary GLC and their relative elution order are shown in Fig. 4. The position of the double bonds in the hexadecenoates was established by GLC of the aldehydes resulting from decomposition of the ozonides (6). \( \Delta \)-2- and \( \Delta \)-3-hexadecenoate isomers gave rise to predominantly tetradeccanal and tridecanal, respectively. Complete hydrogenation of the ynoate esters, allenic ester, and monoenate esters produced only methyl palmitate.

Long chain 2-keto esters were a gift from Dr. Howard Sprecher, Ohio State University. Alkan-2-one standards were provided by Dr. John Gilbertson, University of Pittsburgh. Reduced nucleotides, coenzyme A thioesters, and other incubation components were obtained from Sigma. Lipid standards were purchased from Nu-Chek-Prep, Elysian, MN. All solvents were glass-distilled and were obtained from Burdick and Jackson Laboratories, Muskegon, MI. [\( ^{14} \text{C} \)]Palmitate (55 mCi/mmol) was purchased from Applied Science Laboratories, State College, PA, and purified by TLC before use.

**GLC**—All analyses were carried out on a Varian Aerograph Model 3700 chromatograph equipped with standard and capillary-splitter injectors, digital integrator, and flame ionization detectors. Helium carrier gas was used with all columns. Methyl esters were analyzed on a glass capillary (20 meter \times 0.25 mm (internal diameter)) coated with OV101; a stainless steel capillary (31 meter \times 0.25 mm) coated with diethylene glycol succinate polyester; a glass column (1.54 meter

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1 The abbreviations used are: GLC, gas-liquid chromatography; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; NAC, N-acetylcysteamine.

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Figure 1. A typical HPLC chromatogram showing the resolution of methyl-3-hexadecynoate (peak 1) and methyl-2,3-hexadecadienoate (peak 2) on an octadecyl column.

RESULTS

Identity of Metabolic Products—The unexpected chromatographic behavior and the instability of some of the metabolic products made identification difficult. Unmetabolized acetylenic acid substrates, 16\(\alpha\)=16\(\gamma\) and 18\(\alpha\)=18\(\gamma\), are more polar than normal long chain fatty acids, as shown in Fig. 5, which allowed their separation from the normal free fatty acids. Methyl-2-hexadecynoate exhibited very long GLC retention times on polar columns (1) and even on a nonpolar liquid phase as illustrated in Fig. 4A, peak 11. Unexpectedly, the substrate methyl ester (16\(\alpha\)=16\(\gamma\)) migrated on silver ion TLC with prepurified nitrogen for 20 min before incubations were carried out at 37°C with shaking for 60 min.

Lipid Analyses—Incubations with labeled palmitic acid were stopped with 0.3 ml of 10 \(\mu\)M potassium hydroxide and 2 ml of 95% ethanol. This saponification mixture was heated at 80°C for 1 h, cooled, acidified, and extracted with hexane three times. Methyl esters were prepared (15), aliquots were separated according to degree of unsaturation by silver ion TLC, and the saturated fatty esters were resolved by preparative GLC (16) and collected (17). The radioactivity in each fatty ester was determined in a liquid scintillation counter. The large scale incubations were stopped by the addition of 60 ml of chloroform-methanol (2:1). The chloroform layer was removed, the aqueous layer was washed again with 40 ml of chloroform, the chloroform layers were combined and evaporated, and the lipid extract was analyzed for the fatty acid composition of the individual lipid classes. The CoA thioesters were purified from the aqueous phase using procedures adapted from previous studies (18–20). 25% perchloric acid was added to the aqueous layer and precipitated protein to make a final perchlorate concentration of 5%, which precipitated protein and CoA thioesters. After 1 h at 4°C, the samples were centrifuged, the supernatant was removed, and the precipitate was washed twice with 10 ml of 1% perchlorate, twice with cold ethanol-ether (1:1), and once with cold acetone. The precipitate was dried under a flow of dry nitrogen and the residue was extracted three times with 10 ml of pyridine-2-propanol-water (1:1:1) at 37°C with shaking. The combined extracts containing the CoA thioesters were evaporated to dryness and redissolved in 2 ml of distilled water. These CoA thioesters were saponified, neutralized, and esterified (15) and the methyl esters were used to characterize and identify the acyl moieties using a variety of analytical techniques. Some methylations were made with diazomethane (21).

Total lipids were extracted from microsomal and mitochondrial incubation mixtures by the Bligh and Dyer procedure (22). Individual lipid classes were separated by TLC (23, 24) and methyl esters were prepared (15) and analyzed by GLC. Some methyl ester samples were separated according to degree of unsaturation using argentation TLC and the fractions were analyzed by GLC before and after hydrogenation (25) to facilitate ester identification.

Subcellular Preparations—Cellular fractions were obtained from male Buffalo strain rats (200–250 g) fed a chow diet following the usual procedures (8, 9). Livers were homogenized in four volumes of 0.25 M sucrose, 5 mM tris, 3 mM EDTA buffer at pH 7.4. Cell nuclei and debris were removed by centrifuging at 10 min at 900 \(\times\) g.

Mitochondria were sedimented at 8,500 \(\times\) g for 10 min and washed again with buffer. The mitochondrial supernatant was centrifuged at 15,000 \(\times\) g for 15 min and the resulting supernatant was centrifuged at 100,000 \(\times\) g for 60 min to give the microsomal fraction. The microsomes were washed once with 0.01 M phosphate buffer containing 1 mM EDTA and 1 mM cysteine at pH 7.0. Protein concentration was determined by the biuret reaction (10) using bovine serum albumin as a standard. Marker enzyme activities were measured to estimate purity of the preparations (11). Microsomes contained high levels of NADPH cytochrome c reductase whereas mitochondria contained little or no activity. Cytochrome c oxidase activity, estimated by difference between NADH cytochrome c reductase activities in the presence and absence of cyanide, was high in the mitochondria preparations and very low in the microsomal fractions.

Incubation Conditions—Microsomal fatty acid elongation conditions were adapted from the procedures described by Nugteren (12) and Bernert and Sprecher (13). Mitochondrial fatty acid elongation conditions were adapted from Quagliariello et al. (14). Substrate and cofactor concentrations and buffers used in the incubations are given in the footnotes to Tables I and II. Most incubations were scaled up to 25 to 50 times to produce sufficient quantities of thioester intermediates and products for identification. Incubation flasks were flushed with prepurified nitrogen for 20 min before incubations were carried out at 37°C with shaking for 60 min.

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Reanalysis of the allene and 16=1Δ3 isolated by HPLC by either GLC or silver ion TLC (Fig. 3, lanes 3 and 4) again gave rise to two peaks and two bands, respectively. The results indicate that 16=1Δ3 and 16:2Δ2,3 esters each isomerize to a mixture during GLC and TLC analysis.

The cis- and trans-Δ3 monoenes resulting from the partial hydrogenation of methyl-3-hexadecynoate had identical GLC elution times (Fig. 4, peaks 6 and 7) and RF values on silver ion TLC (Fig. 3, lanes 7 and 8) as two methyl esters isolated from the acyl-CoA thioesters when 16=1Δ2 was a substrate in the incubations. Geometrical isomers of Δ3-octadecenoate were reported to have nearly identical retention times on capillary and packed columns containing polar and nonpolar phases (26, 27). The relative RF values reported for the cis-Δ3 and trans-Δ3 octadecenoates were similar to those reported here for the hexadecenoates (26). The trans isomer resulting from the partial hydrogenation of 16=1Δ2 methyl ester had an identical GLC elution time as a major component from the incubations, when 2-hexadecynoic acid was the substrate (Fig. 4, peak 10). cis-Δ2-Hexadecenoate which eluted much sooner than the trans isomer (Fig. 4, peak 5) was a minor component of the incubations and was not always detectable. The cis-Δ2 isomer migrated even higher than the saturated esters on adsorbent layers impregnated with silver ion whereas the trans-Δ2 isomer migrated only slightly higher than the standard trans isomers (Fig. 3, lanes 5 and 6). The unusual migration behavior of cis-Δ2-octadecenoate has been observed previously (26), but no explanation was offered. Examination of space-filling models shows that the carbonyl oxygen is in close proximity to the allylic hydrogens on carbon four, which permits nonclassical hydrogen bonding and resonance stabilization as depicted in Structure 1. This distribution of the charge renders the electrons unavailable for complexing with the silver ion and stabilizes the conformation. A similar ar-

**FIG. 2.** NMR spectra. A, methyl-2-hexadecynoate; B, methyl-3-hexadecynoate; C, methyl-2,3-hexadecadienoate. The chemical shifts for the number proton resonances are given in the text.
gument can be used to explain the unexpected higher migration of methyl-2-hexadecynoate on TLC. Conjugation of the triple bond electrons with the carbonyl group reduces their availability to complex with the silver ions compared to triple bonds which are removed one or more methylene carbons from the carbonyl such as 16\(\equiv\)13. The intrahydrogen bonding complex of the cis-\(\Delta 2\) isomer is unstable at the temperatures used for GLC, which gives rise to predicted elution times (Fig. 4, peak 5). Analysis by HPLC might be expected to produce early elution times of the cis-\(\Delta 2\) isomers similar to the behavior observed on TLC. The trans-\(\Delta 2\) isomer cannot form such a stabilized structure and thus behaves as the standard trans fatty esters on silver ion TLC (Fig. 3, lane 6).

**Effect on Elongation—**The effect of 16\(\equiv\)1\(\Delta 2\) on the elongation of palmitate to stearate in the mitochondrial and microsomal preparations is shown in Table I. Comparisons of the radiolabeled stearate formed when palmitate or 16\(\equiv\)1\(\Delta 2\) was added to the incubations indicate the microsomal elongation system was inhibited dramatically by the acetylenic acid.

**Types of Thioesters—**The relative amounts of acyl, \(\beta\)-ketoacyl-, and \(\beta\)-hydroxyacyl-CoA thioesters formed in the elongation systems when various substrate and incubation conditions were used are given in Table II. \(\beta\)-Keto or \(\beta\)-hydroxy esters were never detected in microsomal or mitochondrial blanks and only small percentages of \(\beta\)-hydroxy esters were detected when oleate was used as the substrate. Normal acyl and \(\beta\)-ketoacyl esters were the only products formed when either 16\(\equiv\)1\(\Delta 2\) or 18\(\equiv\)1\(\Delta 2\) fatty acids were incubated with microsomes without reduced nucleotides. The microsomal incubations always contained a higher percentage of \(\beta\)-keto esters, even when reduced nucleotides were present, than did the mitochondria. In contrast, mitochondria incubated with the acetylenic acids produced both \(\beta\)-keto and \(\beta\)-hydroxy esters when reduced nucleotides were omitted from the system. \(\beta\)-Ketoacyl thioesters were reduced to a very low level in the mitochondria when reduced nucleotides were included in the incubations.

**Composition of Thioesters—**Analysis by GLC of the methyl esters derived from the \(\beta\)-hydroxyacyl thioesters revealed the presence of only the \(\beta\)-hydroxy acids of the same chain length as the added substrate: 18:1\(\Delta 9\), 16\(\equiv\)1\(\Delta 2\), and 18\(\equiv\)1\(\Delta 2\) gave rise to \(\beta\)-OH 18:1\(\Delta 9\), \(\beta\)-OH 16:0, and \(\beta\)-OH 18:0, respectively. Long chain alkan-2-ones, commonly referred to as methyl ketones, derived from the \(\beta\)-ketoacyl thioesters, were analyzed by GLC. Only methyl ketones of one carbon less than the substrate chain length were detected: 16\(\equiv\)1\(\Delta 2\) and 18\(\equiv\)1\(\Delta 2\) gave rise to 15:0 and 17:0 methyl ketones, respectively. The saponification of \(\beta\)-ketoacyl-CoA esters has previously been shown to yield methyl ketones (12, 13).

Methyl esters derived from the acyl-CoA thioesters were analyzed by GLC on a polar packed column and the data are shown in Table III. When the 16\(\equiv\)1\(\Delta 2\) was the substrate in

![Fig. 3. Thin layer chromatoplate of Silica Gel G impregnated with silver ions.](image)

![Fig. 4. Comparisons of methyl esters prepared from microsomal CoA thioesters.](image)
the incubations of both subcellular preparations, two new esters that were not present in the control incubations were observed in large amounts. One of the esters was identified as the acetylenic acid substrate, indicating that it had been activated. The other peak was identified as trans-Δ2-hexadecenoate. In contrast to incubation with 16ΔΔ2, when oleate was the substrate, it represented more than 70% of the CoA thioesters in both subcellular preparations. Although not shown in Table III, oleate was elongated to 20:1 which represented one to 2.5% of the total. Eicosenoate was not detected in the esters from the controls. The absence of 16=1ΔΔ2 and 16ΔΔ2 CoA thioesters when boiled subcellular preparations were incubated with the acetylenic acid indicates that the trans-Δ2-hexadecenoate was not an artifact of sample preparation and that 2-hexadecynoate did not result from substrate carryover during acetyl-CoA thioester isolation. The decrease of polyunsaturated fatty acids in the boiled controls probably resulted from oxidation.

The methyl esters derived from the acetyl-CoA thioesters were also examined by capillary GLC. Typical chromatograms of methyl esters from microsomal incubations with 16ΔΔ2 (A) and control incubations (B) are shown in Fig. 4. Quantitative data on the C-16 and C-18:1 fatty esters isolated from acetyl-CoA thioesters of the various incubations are shown in Table IV. The changes in the proportions of the major octadecenoates were minimal under all incubation conditions. The differences between the hexadecenoate content of control microsomal and mitochondrial incubations were also minimal. Methyl-2-hexadecynoate, the substrate, always represented a higher percentage of the C-16 esters in the mitochondria than in the microsomal system. trans-Δ2-Hexadecenoate was the major hexadecenoate in both mitochondria and microsome acyl-CoA thioesters when the acetylenic acid was the substrate. In addition to the Δ6, Δ7, and Δ9 hexadecenoates which were present in control incubations, cis-Δ3- and trans-Δ3-hexadecenoates and Δ2,3-hexadecadienoate were also identified in the CoA thioesters of the incubations with acetylenic acid.

**Effect on Cellular Lipids**—The effect of the 2-hexadecynoate

**Table I**

| Subcellular fraction | Incubation conditions* | Palmitate elongated to stearate cpm/mg protein/min |
|----------------------|------------------------|-----------------------------------------------|
| Mitochondrial        | Complete, no additions | 83.6                                          |
|                      | Complete + 0.1 mM 16:0 | 28.5                                          |
|                      | Complete + 0.1 mM 16 = 1ΔΔ2 | 26.7                                      |
| Microsomal           | Complete, no additions | 97.8                                          |
|                      | Complete + 0.1 mM 16:0 | 32.1                                          |
|                      | Complete + 0.1 mM 16 = 1ΔΔ2 | 9.0                                           |

* The complete system consisted of 10 μmol of ATP, 2 μmol of NADPH, 1 μmol of CoA, 10 μmol of MgCl₂, 0.86 μmol (1 × 10⁻²⁶/cpm) of [1-1³C]palmitic acid in 2 ml of 0.4 μmol of phosphate buffer (pH 7.4). In addition, the mitochondrial system contained 1 μmol of NADH, 1 μmol of acetyl-CoA, and 2 mg of protein, and the microsomal system contained 1 μmol of malonyl-CoA and 5 mg of protein. Incubations were carried out for 30 min at 37 °C under nitrogen in a final volume of 2 ml.

**Table II**

| Substrates and elongation system | Acyl | β-Ketoacyl | β-Hydroxyacyl |
|----------------------------------|------|------------|---------------|
| Incomplete system* |       |            |               |
| Blanks (mito. and micro.)* | 100  | 0          | 0             |
| 18=Δ9C, micro. | 96.9 | 0          | 3.1           |
| 16 = ΔΔ2, micro. 1 | 39.8 | 60.2      | 0             |
| 16 = ΔΔ2, micro. 2 | 47.0 | 53.0      | 0             |
| 18 = ΔΔ2, micro. | 53.1 | 46.9      | 0             |
| 18=Δ9C, micro. | 93.5 | 0          | 6.5           |
| 16 = ΔΔ2, micro. 1 | 72.2 | 20.2      | 7.6           |
| 16 = ΔΔ2, micro. 2 | 84.1 | 13.2      | 2.7           |
| 18 = ΔΔ2, micro. | 90.7 | 5.9       | 3.3           |
| Complete system* |       |            |               |
| 16 = ΔΔ2, micro. 1 | 83.4 | 4.3       | 12.3          |
| 16 = ΔΔ2, micro. 2 | 78.1 | 15.8      | 6.2           |
| 16 = ΔΔ2, micro. 1 | 95.4 | 1.0       | 3.6           |
| 16 = ΔΔ2, micro. 2 | 91.0 | 1.1       | 7.9           |

* The incomplete incubation system contained 500 μmol of ATP, 30 μmol of CoA, 500 μmol of glutathione, 500 μmol of MgCl₂, buffered at pH 7.4 in 5 mmol of potassium phosphate, 50 μmol of substrate were suspended in 4 ml of 1% Triton WR 1339 by sonication. Flasks were flushed with nitrogen for 20 min before incubating at 37 °C for 1 h with 120 mg of protein. Mitochondrial incubation also contained 50 μmol of MnCl₂. Final incubation volume was 40 ml.

* Mitochondrial (mito.) and microsomal (micro.) blanks, consisting of boiled protein or extracted at time zero, from complete, or incomplete incubation systems, were all similar.

* The complete system contained, in addition to the components of the incomplete system, 25 μmol of malonyl-CoA and 60 μmol of NADPH in the microsomes and 25 μmol of acetyl-CoA, 60 μmol of NADPH, 60 μmol of NADH, and 50 μmol of MnCl₂ in the mitochondria.
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**TABLE III**

Fatty acid composition of acyl-CoA thioesters from mitochondrial and microsomal elongation systems

See text for incubation conditions and substrate concentrations. Values with standard deviations represent two separate experiments. All other values are from single experiments. The difference between the sum of the percentages in any row and 100 represents the sum of other minor components not shown.

| Preparation source and incubation conditions | Fatty acid |
|---------------------------------------------|------------|
|                                             | 14:0 | 16:0 | 16:1* | 16:1Δ2 | 16 = 1Δ2 | 17:0 | 18:0 | 18:1 | 18:2 | 20:4 |
| Mitochondrial                               | %    | %    | %    | %    | %    | %    | %    | %    | %    | %    |
| Mito., no substrate, no incubation          | 0.7  | 15.7 | 1.9  | 1.7  | 35.8 | 10.9 | 19.5 | 10.7 |
| Boiled mito. + 16 = 1Δ2                      | 2.9  | 35.2 | 4.2  | 4.4  | 37.9 | 6.8  | 3.5  | 1.1  |
| Mito. + oleate                               | 3.3  | ±0.1 | 18.7 | ±0.1 | 3.8  | ±2.1 | 9.0  | ±7.9 | 19.4 | ±3.5 | 0.8  | ±0.4 | 16.7 | ±0.8 | 4.6  | ±1.0 | 8.0  | ±4.7 | 14.4 | ±2.2 |
| Microsomal                                  | 0.3  | 16.9 | ±0.1 | 1.3  | ±0.1 | 1.4  | ±0.4 | 27.0 | ±0.1 | 8.0  | ±0.1 | 15.3 | ±2.1 | 24.9 | ±0.1 |

* The composition of the C-16 fraction is shown in Table IV.

**TABLE IV**

C-16 and 18:1 composition of mitochondrial and microsomal acyl-CoA thioesters before and after incubation with 2-hexadecynoic acid

The concentration of acyl moieties in the CoA thioesters isolated from mitochondrial (mito.) and microsomal (micro.) controls and the corresponding incubations with substrate (Experiment 2) were 3.1, 13.0, 6.2, and 13.5 μg/mg of protein, respectively. Based upon the data in Table III and this table, substrate and metabolites represented 15–25% and 25–35% of the total fatty acyl-CoA esters isolated from microsomal and mitochondrial incubations, respectively. See text for incubation conditions.

| Preparation source and incubation conditions | C-16 | C-18:1 |
|---------------------------------------------|------|-------|
|                                             | 16:0 | 16 = 1Δ2 | 16:1Δ2 | 16:1cis-Δ3 | 16:1trans-Δ3 | 16:1cis-Δ6 + Δ7 | 16:1cis-Δ9 | 16:2Δ2, 3 | cis-Δ9 | cis-Δ11 |
| Mitochondrial                               | %    | %     | %     | %     | %      | %       | %        | %        | %      | %     |
| Mito., no substrate, no incubation          | 92.2 | 0.4   | 7.4   | 43.5  | 56.5   |
| Boiled mito. + 16 = 1Δ2                     | 91.0 | 3.9   | 5.1   | 65.1  | 34.9   |
| Mito., 16 = 1Δ2 (Experiment 1)              | 41.6 | 45.2  | 7.7   | 0.4   | 2.6    | 0.2     | 1.3      | 1.0      | 63.3   | 36.7   |
| Mito., 16 = 1Δ2 (Experiment 2)              | 34.7 | 27.9  | 25.7  | 2.3   | 6.1    | T*      | 0.9      | 2.4      | 54.7   | 45.3   |
| Microsomal                                  | %    | %     | %     | %     | %      | %       | %        | %        | %      | %     |
| Mito., no substrate, no incubation          | 95.8 | 0.9   | 3.3   | 54.5  | 45.5   |
| Boiled mito. + 16 = 1Δ2                     | 93.0 | 3.2   | 3.8   | 79.4  | 20.6   |
| Mito., 16 = 1Δ2 (Experiment 1)              | 62.5 | 10.3  | 17.0  | 1.2   | 5.9    | 0.4     | 2.7      | T        | 73.8   | 26.5   |
| Mito., 16 = 1Δ2 (Experiment 2)              | 43.3 | 2.6   | 32.7  | 2.6   | 3.6    | 0.2     | 2.2      | 12.3     | 61.0   | 39.0   |

* T denotes amounts too small to quantitate.

noate on the quantity of the major lipid classes and the fatty acid composition of the lipid classes in the microsomes and mitochondria relative to control preparation was minimal. The quantities of diphasphatidylglycerol (25–30% of total phosphorus) in control and 16=1Δ2 -treated mitochondria, relative to the percentage (5%) in microsomal preparations, lend additional support to the purity of the subcellular preparations. Microsomes were characterized by a high percentage of phosphatidyserine + phosphatidyl ethanolamine (≈20%) and sphingomyelin (=10%). A minor unidentified component with an RF value similar to diphasphatidylglycerol was the only phospholipid that contained either the substrate or metabolic product. Neutral lipid classes from control of 16=1Δ2 -treated mitochondria or microsomes were similar, as shown in Fig. 5. Analysis of the free fatty acids, which were completely resolved from the 16=1Δ2 substrate (Fig. 5), showed only traces of the metabolic products: 16:1Δ2t, 16:1Δ3c, and 16:1Δ3t. Cholesterol ester, a minor component (Fig. 5), was the only neutral lipid class that contained esterified acetylenic acid substrate, but the percentage was less than 5% of the total. Longer incubation times or analysis of liver microsomes and mitochondria from animals fed dietary 1-1A2 will be required to determine whether substrate or metabolic products are incorporated to any significant extent into complex lipids.

**DISCUSSION**

Data from these in vitro studies confirm earlier data from intact animals that indicated 2-hexadecynoate inhibited fatty acid elongation (I). Under the conditions employed, the microsomal system appeared to be inhibited preferentially. The enzymes responsible for metabolism of the 2-ynoic acids appear to be present in both the mitochondria and microsomes. The proposed pathway for the metabolism of acetylenic fatty acids, inhibitor production, and one site of inhibition is given in Fig. 6. The 2-ynoic acid (I) is converted to the CoA ester (II), presumably by long chain acyl-CoA synthetase.
(A). Identification of methyl-2-hexadecynoate (Tables III and IV) in the CoA esters from both subcellular preparations was conclusive. These data agree with the reported formation of dec-3-ynoic-CoA in rat liver microsomes (28). An isomerase (B) acted on the acetylenic acyl-CoA (II) to produce a 2,3-allene (III) which thermally isomerized to elute as a mixture of 2,3-allene and 3-hexadecynoate on capillary GLC (Fig. 4). An isomerase that yields 2,3-allene from 3-ynoate thioesters has been known for some time, but the isomerization of 2-ynoate does not appear to have been reported previously. A series of papers by Bloch and his colleagues (4, 29, 30) has demonstrated that the NAC thioester of dec-3-ynoic acid is enzymatically converted by E. coli &beta; hydroxydecanoyl thioester dehydrase to a 2,3-allene that reacts instantly, forming a covalent linkage with the enzyme resulting in irreversible inhibition. More recently, Miesowicz and Bloch (31) have isolated and characterized an isomerase from hog liver that catalyzes the reversible isomerization of 3-ynoate-NAC to the (+)-2,3-allene. This isomerase does not appear to be the enzyme that catalyzes reaction B in Fig. 6 because they reported oct-2-ynoic-NAC was not isomerized to the 2,3-allene. However, the inactivity of the hog liver isomerase on the 2-ynoate may have resulted from the use of a short chain substrate or the use of the NAC derivative. Whether the acetylene-allene isomerases of rat liver (step B, Fig. 6) and hog liver (31) are the same remains to be established, but the allenic products appear to be the same.

Whether the hydration of the 2-ynoate (II) at reaction C (Fig. 6) or the hydration of the allene (III) at reaction D, or both, proceeds to yield the &beta;-keto ester (V) after rearrangement cannot be established from our data. Although enzymatic activities were low, the hydration of a 2,3-allene to the &beta;-ketoacyl ester by enoyl-CoA hydrolase has been observed (32). The &beta;-ketoacyl esters (V), corresponding to the chain length of the acetylenic acid, were found to accumulate in the incubations, especially in the microsomes when reduced nucleotides were omitted (Table II). The &beta;-hydroxyacyl-CoA esters (VI) resulting from the reduction of &beta;-keto esters (reaction F) were identified in both subcellular preparations when reduced nucleotides were in the incubations (Table II). trans-Δ2-Acyl-CoA (VII), resulting from the dehydrogenation of the &beta;-hydroxyacyl ester (reaction G), accumulated (Table IV), indicating the inhibition of enoyl-CoA reductase (reaction H). Based upon the concentration of the various intermediates in the proposed pathway and the partial structural similarity of the 2,3-allene (III) to trans-Δ2-CoA ester (VII), it is proposed that the 2,3-allene is an inhibitor of enoyl-CoA reductase. Obviously, the CoA ester of the acetylenic acid may also act as an inhibitor of this enzyme.

The relatively low level of the 2,3-allene (III) in the CoA esters under most incubation conditions (Table IV) may indicate the allene is being metabolized further. This view is supported by the identification of cis- and trans-Δ3 hexadecenoates (X and IX) (Table IV and Fig. 4), indicating the 2,3-allene is reduced (reaction K). The isomerization of the cis- and trans-Δ3-acyl-CoA esters to the trans-Δ2-acyl-CoA ester (VII) (reaction L) has been shown to be catalyzed to hog liver isomerase (31), a second function of the acetylene-allene isomerase. Enoyl-CoA hydratase has been also shown to catalyze the isomerization of the Δ3 geometrical isomers to the trans-Δ2 isomer (33).

Studies with propynyl-CoA and but-2-ynoic-CoA have been reported to inhibit fatty acid biosynthesis in vitro (34). Specifically, the condensation of malonyl-CoA with acetyl-CoA and the reduction of crotonyl-CoA to butyrate were inhibited, whereas the reduction of acetoacetyl-CoA to β-hydroxybutyrate was not inhibited (34). The latter two observations are consistent with reactions H and F of Fig. 6. The 2-hexadecynoic-CoA or the 2,3-allenoyl-CoA esters may inhibit the elongation of fatty acids by inhibiting the condensation of malonyl-CoA with long chain acyl-CoA. Cytoplasmic thiolase has been shown to be inhibited by short chain 3-ynoic acids (28), but our studies with intact animals fed methyl-2-hexadecynoate did not contain lower sterol levels (1). Likewise, our studies with intact animals did not indicate fatty acid biosynthesis was inhibited as suggested from in vitro studies with the short chain 2-ynoic-CoA esters (34).

The present study indicates that a metabolic product of 2- and 3-acetylenic fatty acids inhibits fatty acid elongation at the last step in the pathway. Indirect evidence also suggests that long chain 2-hexadecynoyl- or allenoyl-CoA esters may inhibit the microsomal, and perhaps the mitochondrial, fatty acid elongation systems at another site, namely the condensation reaction.

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