Biochemical Properties of Cytochrome \( b_5 \)-dependent Microsomal Fatty Acid Elongation and Identification of Products*

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Recently, our laboratory reported the involvement of cytochrome \( b_5 \) in the elongation of hepatic microsomal fatty acids (Keyes, S. R., Alfano, J. A., Jansson, I., and Cinti, D. L. (1979) J. Biol. Chem. 254, 7778-7784). In this paper, a correlation between the rates of \( b_5 \) reoxidation in the presence of malonyl-CoA and malonyl-CoA incorporation measured under various conditions was demonstrated, thus providing further evidence in support of our hypothesis.

The additional data in this paper detail the biochemical characteristics of the microsomal fatty acid elongation reaction. The rates of \( b_5 \) reoxidation and malonyl-CoA incorporation were linear with protein concentration up to 2 mg/ml and were optimal between pH 7.2 and 3.4. The rates were linear with malonyl-CoA concentration up to 3 \( \mu \)M and were maximal at 20 \( \mu \)M with a \( K_m \) of 8 \( \mu \)M. The \( b_5 \) reoxidation rate was always 10 to 15% faster than the incorporation rate.

The products formed during fatty acid elongation were 18:0 (15%), 18:1 (65%), and 20:1 (20%) using either NADH or NADPH as the reducing agent. The rates of incorporation were 30% faster with NADPH than with NADH, although the extent of incorporation was nearly identical. The apparent \( K_m \) for NADH and 6 \( \mu \)M for NADPH.

The 18:1 formed during malonyl-CoA incorporation was 60% oleic acid and 40% vaccenic acid as demonstrated by ozonolysis. Both palmitic and palmitoleic acids were substrates for the malonyl-CoA incorporation reaction and the elongation rates of the two fatty acids were 60% and 40%, respectively, of the rate of elongation utilizing the endogenous microsomal fatty acids. The formation of 18:1 using endogenous fatty acids was partially, but not completely, blocked by 1 mM KCN as would be expected from the composition of the 18:1 formed during malonyl-CoA incorporation. Finally, the rates of \( b_5 \) reoxidation and malonyl-CoA incorporation and product formation were unaffected by carbon monoxide.

In the early 1960's, several investigators demonstrated the elongation of liver microsomal fatty acids in the presence of malonyl-CoA, ATP, and reducing equivalents in the form of NADH or NADPH (1-3). Furthermore, elongation was shown to occur in the absence of ATP, provided a fatty acyl-CoA was added to the reaction mixture (4). These data suggested that the coenzyme A derivatives of the endogenous fatty acids were being formed via the ATP-requiring fatty acyl-CoA ligase (5) utilizing the CoASH released from malonyl-CoA.

Although the general outline of microsomal elongation has been well documented, no systematic study of the reaction, quantifying the apparent \( K_m \) for substrates and cofactors, pH optimum, and product identification has been performed.

The general mechanism of microsomal and mitochondrial fatty acid elongation and \( de novo \) fatty acid synthesis has been shown to be similar for each biosynthetic reaction (4, 6, 7): condensation of the preferred substrates to form a \( \beta \)-keto, reduction of the \( \beta \)-keto, to a secondary alcohol, dehydration of the alcohol to form a trans-\( \beta \)-double bond and reduction of the trans \( \beta \)-double bond (Fig. 1).

Recently, our laboratory implicated a role for cytochrome \( b_5 \) in the NADH-dependent elongation of microsomal fatty acids (11). We found that the rate of \( b_5 \) reoxidation increased in the presence of malonyl-CoA plus ATP and that malonyl-CoA incorporation was inhibited by an anti-cytochrome \( b_5 \) IgG. In addition, the malonyl-CoA-enhanced \( b_5 \) reoxidation rate was significantly lowered if the microsomal \( b_5 \) content was decreased to the extent that cytochrome \( b_5 \) became rate-limiting (12). Hence, as indicated in Fig. 1, cytochrome \( b_5 \) is postulated to accept electrons from either microsomal flavoprotein reductase and to transfer these electrons to the enzyme components in one or both of the reduction steps.

Bernet and Sprecher (13) have recently reported that the condensation reaction was the rate-limiting step for microsomal fatty acid elongation with palmitoyl-CoA as the preferred substrate, which was converted to stearic acid. When endogenous fatty acids have been used as substrates for fatty acid elongation, stearic acid was reported to be the primary product (3, 14).

The data, presented in this paper, detail the biochemical properties of the hepatic microsomal fatty acid elongation

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were given access ad libitum to a high carbohydrate, fat-free diet and NaCl. Microsomes were obtained by differential centrifugation in 0.25 M sucrose and by Ca2+ precipitation (15). The final microsomal pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing 0.15 M KCl. The protein concentration was measured by the biuret method (16).

Measurement of Cytochrome b, Reoxidation Rates—The reaction mixture used for measuring cytochrome b6 reoxidation rates in a spectrophotometric cuvette consisted of the following components (final concentration): 100 mM Tris-HCl, pH 7.4, 2 μM rotenone, 500 μM ATP, and 1 mg/ml of liver microsomes, which were equilibrated to 37°C. The complete reduction of cytochrome b6 was initiated with NADH (3 μM), following its addition to the cup of a plunger which was placed into the cuvette. All other additions (indicated in figure legends) were included with NADH in the plunger, giving a final reaction volume of 2.5 ml.

The rate of reoxidation of reduced cytochrome b6 was determined by measuring the difference in absorbance between 424 nm and 410 nm in an Aminco DW-2 UV/VIS spectrophotometer in the dual wavelength mode; the extinction coefficient used was 185 mm-1 cm-1 (17). The substrate-stimulated b6 reoxidation rate was reported as the difference between the rate in the presence and absence of substrate.

Malonyl-CoA Incorporation—The assay mixture for measuring microsomal fatty acid elongation contained the following components (final concentration): either 20 μM (50,000 cpm) [1,3-14C]malonyl-CoA or 20 μM (25,000 cpm) [2-14C]malonyl-CoA, 2 μM rotenone, 500 μM ATP, 1 mM reduced pyridine nucleotide, and 100 mM Tris-HCl, pH 7.4. Following a 5-min preincubation period at 37°C, the reaction was initiated with 100 μl of liver microsomes (1 mg/ml of final concentration), giving a total volume of 1.25 ml. At the end of the incubation time, the reaction was stopped with 0.5 ml of 15% KOH/methanol. The isolation, methylation, and identification of fatty acids were performed as described previously (11), with the following exceptions: a Varian-Aerograph 3700 gas-liquid chromatograph (GLC) was used with a column of 10% silar 10C (silicone) on 100/120 Gas-Chrom Q (stainless steel, ½ inch outer diameter x 6 feet, Applied Science, State College, Pa.). The column temperature was programmed at 140°C for 35 min, then increased at a rate of 20°C/min to 220°C, which was held for 5 min. The flow rate was 45 cm/min. A Packard model 894 gas proportional counter, attached to the Varian GLC was used to measure the radiolabel in individual methylated fatty acids.

Data Analysis—The Km values for NAD(P)H and malonyl-CoA were obtained from Lineweaver-Burk plots, using data points which had been fitted to a straight line by a least squares linear regression program. The calculated Km values were accepted if the correlation coefficient was greater than 0.99. Standard deviations were reported if the sample number was greater than or equal to 3.

Oxidolysis of Radiolabeled Fatty Acids—Following extraction of fatty acids from the microsomes used in the malonyl-CoA incorporation assay, the organic solvent was evaporated under a stream of nitrogen. The fatty acids were then methylated (18) with 1 ml of BF3/MEOH by heating in a boiling water bath for 2 min. The reaction was stopped by the addition of 1 ml of deionized distilled water and the fatty acid methyl esters were extracted twice with 2.5 ml volumes of n-hexane.

Oxidolysis was performed with the Supelco Kit (Bellefonte, Pa.) by passing ozone (generated by exposing oxygen to a Tesla coil) through the fatty acid methyl ester mixture for 1 min, while keeping the reaction mixture at ~60°C to reduce side reactions. Next, the mixture was flushed with nitrogen and then a small amount (~1 mg) of triphenylphosphine was added and blended on a Vortex mixer. The aldehyde esters, formed by the oxidative cleavage of the radiolabeled fatty acid methyl esters, were identified using radio-gas liquid chromatography by comparison with the products of ozonized oleic acid methyl ester and vacenonic acid methyl ester.

Chemicals—NADH, NADPH (enzymatically reduced), malonyl-CoA, ATP, oleic acid, and stearoyl-CoA were obtained from Sigma (St. Louis, Mo.); palmitoyl-CoA and palmitoleoyl-CoA from Avanti Biochemicals (Birmingham, Ala.) and vacenonic acid from Supelco Co., Inc. (Bellefonte, Pa.). All radio labeled chemicals were purchased from New England Nuclear (Boston, Mass.).

RESULTS

Establishment of Controls for the Malonyl CoA Incorporation Assay

When using [1,3-14C]malonyl CoA for incorporation experiments, it was noticed that a substantial amount of radioactivity was extracted, if NADH was omitted from the reaction system, including the pH optimum and the apparent Km for malonyl-CoA, NADH, and NADPH. In addition, a comparison of the methods of malonyl-CoA incorporation and of b6 reoxidation, used in determining the biochemical characteristics of fatty acid elongation, indicated that both methods could be used interchangeably for studying hepatic microsomal fatty acid elongation. Finally, under our assay conditions, we observed that the predominant product of microsomal elongation as determined by radio-gas liquid chromatography was octadecenoic acid (18:1) which was composed of 60% oleic acid (9-18:1) as identified by ozonolysis and by the distribution of the products of malonyl-CoA incorporation in the presence of potassium cyanide.

MATERIALS AND METHODS

Isolation of Microsomes—Male Sprague-Dawley rats, 150 to 200 g, were given access ad libitum to a high carbohydrate, fat-free diet (L.C.N., Cleveland, OH) for 1 to 4 days, after an initial 24-h starvation period. All animals were killed by decapitation between 8 and 9 a.m., and the livers were quickly removed and perfused with ice-cold 0.9% NaCl. Microsomes were obtained by differential centrifugation in 0.25 M sucrose and by Ca2+ precipitation (15). The final microsomal pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing 0.15 M KCl. The protein concentration was measured by the biuret method (16).

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Liver Microsomal Fatty Acid Elongation

Fig. 2. Malonyl-CoA incorporation into microsomal fatty acids using either [1,3-14C]malonyl-CoA or [2-14C]malonyl-CoA in the presence or absence of NADH. The reaction mixture contained 2 μm rotenone, 500 μM ATP, 20 μM radiolabeled malonyl-CoA, 0.1 M Tris-HCl, pH 7.4, and microsomes (1 mg/ml) from rats on a fat-free diet in a total volume of 1.25 ml. The reaction mixture was incubated at 37°C for 3 min. A, [1,3-14C]malonyl-CoA (50,000 cpm); B, [1,3-14C]malonyl-CoA + 1 mM NADH; C, [2-14C]malonyl-CoA (25,000 cpm); D, [2-14C]malonyl-CoA + 1 mM NADH. Smooth line, gas liquid chromatograph trace; jagged line, radioactive trace.

De novo fatty acid synthesis was also measured using the

standard assay conditions with the addition of either NADH or NADPH, radiolabeled acetyl-CoA, and unlabeled malonyl-CoA. The maximal activity under these conditions was 3% and 8% of malonyl-CoA incorporation with NADH and NADPH, respectively. This contamination would not contribute significantly to the microsomal fatty acid elongation and examination of the products of elongation showed little or no label in 16:0 (palmitic acid), the major product expected from de novo fatty acid synthesis in liver (19).

Biochemical Properties of Microsomal Fatty Acid Elongation

Time Course—In order to determine the linearity of the rate of malonyl-CoA incorporation into endogenous fatty acids, a time course for malonyl-CoA incorporation in the presence of NADH or NADPH was performed. Malonyl-CoA incorporation was linear for 3 or 5 min with NADPH or NADH, respectively (Fig. 3). As a consequence, a 2- or 3-min
Liver Microsomal Fatty Acid Elongation

FIG. 3. Effect of incubation time on [2-14C]malonyl-CoA incorporation into microsomal fatty acids with NADPH or NADH. The incubation conditions were described in Fig. 2. ●—●, 1 mM NADPH; ○—○, 1 mM NADH.

FIG. 4. Effect of protein concentration on malonyl-CoA-enhanced \( b_5 \) reoxidation rates and incorporation rates of [2-14C]malonyl-CoA in the presence of NADH. Both reactions contained the components listed in Fig. 2 except the volume for the \( b_5 \) reoxidation assay was 2.5 ml. The incubation time for the malonyl-CoA incorporation assay was 3 min. ●—●, rate of \( b_5 \) reoxidation with malonyl-CoA; ○—○, rate of [2-14C]malonyl-CoA incorporation.

The reason for the faster rate of \( b_5 \) reoxidation (after correction for the endogenous rate) is not known, although it may reflect the measurement of other reactions besides elongation that involve cytochrome \( b_5 \). For example, the formation of products, such as palmityl-CoA and stearoyl-CoA, would result in their desaturation by the cytochrome \( b_5 \)-dependent \( \Delta_7 \)-desaturase.

Malonyl-CoA Concentration and pH Dependence—Varying the malonyl-CoA concentration resulted in a linear increase to approximately 3 \( \mu \)M for both malonyl-CoA incorporation and \( b_5 \) reoxidation, with maximal activity obtained at 20 \( \mu \)M (Fig. 5). As was observed with protein concentration \( b_5 \), reoxidation rates were 10 to 15% faster than the incorporation rates. The apparent \( K_m \) values for the malonyl-CoA obtained from Lineweaver-Burk plots of the enhanced \( b_5 \) reoxidation rates and for the malonyl-CoA incorporation were 4.7 ± 1.4 \( \mu \)M and 8.5 ± 1.0 \( \mu \)M, respectively, and were of the same order of magnitude.

As seen in Fig. 6, the rates, measured by either incorporation or \( b_5 \) reoxidation, were virtually 0 at pH 6. As the pH of the reaction mixture was increased, the rates reached an optimum between pH 7.2 and 7.4. Above pH 7.4, both reaction rates decreased, with a loss of 70% of the optimal activity at pH 8.5. Below the pH optimum, the \( b_5 \) reoxidation rates were again 10 to 15% faster than the malonyl-CoA incorporation rates and between pH 7.5 and 8.0 both rates were virtually identical, suggesting, as indicated earlier, the inhibition of a cytochrome \( b_5 \)-dependent reaction that was not related to elongation.

Pyridine Nucleotide Co-factor Requirements

As alluded to earlier, only the condensation step of the fatty acid elongation reaction proceeded in the absence of a reduced pyridine nucleotide. The rate of malonyl-CoA incorporation using NADPH was 30% faster than the rate using NADH (Table II). In the presence of both pyridine nucleotides, the rate of incorporation was neither additive nor synergistic, but rather the mean of the sum of the two activities, suggesting a competition for the same enzyme system. Furthermore, the percentage of each of the major products was not significantly different. With either pyridine nucleotide, the predominant

incubation period was used for experiments with NADPH or NADH, respectively. It was also noted that the initial rate of malonyl-CoA incorporation with NADPH as the cofactor was always slightly faster (20 to 30%) than the rate obtained with NADH; however, over this time range the extent of incorporation was not significantly different (Fig. 3). Both reactions appeared to proceed in a parallel manner toward completion.

Protein Concentration—Fig. 4 depicts the malonyl-CoA incorporation rates and malonyl-CoA-stimulated \( b_5 \) reoxidation rates as a function of protein concentration. Both rates were linear over the protein concentration range of 0.5 to 2.0 mg/ml, although the \( b_5 \) reoxidation rate was 10 to 15% faster than the rate of incorporation of radiolabeled malonyl-CoA.
vaccenic acid.

fatty acids were employed as the substrates for elongation and
elongation of palmitic acid to stearic acid, followed by desat-
uration; the radioactivity was associated with
incubation period.

The radioactivity was associated with the elongation system does not have a
preference for either reducing agent.

Radiolabeled fatty acid was used, ATP was omitted.

d Radiolabeled products were not detected by the proportional counter.

The data suggest that the 18:1 product represents a mixture of two fatty acids—vaccenic acid (11-18: 1) formed from palmitoyl-CoA and oleic acid (9-18:1) formed from palmitoyl-CoA.

Identification of the Products of Microsomal Fatty Acid Elongation

In all previous experiments, the endogenous microsomal fatty acids were employed as the substrates for elongation and under these conditions the major product formed was 18:1. This product could be formed by two possible pathways: (a) elongation of palmitic acid to stearic acid, followed by desaturation to oleic acid, or (b) elongation of palmitoleic acid to vaccenic acid.

In order to investigate the pathways for biosynthesis of 18:1, the elongation of exogenous fatty acids was studied by substitution of ATP in the standard assay with appropriate fatty acyl-CoA substrates, as ATP was only necessary for the generation of the CoA derivative. As shown in Table III, both palmitoyl-CoA and palmitoleyl-CoA were converted predominantly to 18:1 and approximately 5 to 16% of a C20 fatty acid was formed from both substrates. In addition, palmitoyl-CoA was converted to 12% 18:0, and the product distribution was similar to that found employing endogenous fatty acids as substrate. The rate of elongation of palmitoyl-CoA was faster than that of either palmitoleyl-CoA or stearoyl-CoA, but slower than the elongation rate obtained with the endogenous fatty acids (Table III). Interestingly, the sum of the rates of palmitoyl-CoA and palmitoleyl CoA elongation equaled the elongation rate of the endogenous fatty acids (0.81 + 0.54 equals the endogenous rate). Based on these rates, one can calculate that a minimum of 40% of the 18:1 is derived from 16:1, whereas a maximum of 60% of the 18:1 is derived from 16:0 via 18:0. Hence, the data suggest that the 18:1 product represents a mixture of two fatty acids—vaccenic acid (11-18: 1) formed from palmitoyl-CoA and oleic acid (9-18:1) formed from palmitoyl-CoA.

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radiolabeled fatty acid was 18:1; approximately 7 to 17% of the radioactivity was associated with 18:0 and the remaining 15% with C20 fatty acids. The apparent $K_a$ values for NADH and NADPH were 7.19 ± 3.18 µM and 8.14 ± 1.87 µM, respectively, suggesting that the elongation system does not have a preference for either reducing agent.

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Liver Microsomal Fatty Acid Elongation

**Fig. 7.** Ozonolysis of the radiolabeled 18:1 formed during the [2-14C]malonyl-CoA incorporation assay in the presence of NADH. The incubation conditions were described in Figs. 2 and 4, and the ozonolysis procedure was described under "Materials and Methods." A, radiolabeled products before ozonolysis. B, radiolabeled products after ozonolysis, smooth line, gas liquid chromatograph trace; jagged line, radioactive trace.

**TABLE IV**

Effect of carbon monoxide on the products and rate of [2-14C]-malonyl-CoA incorporation

<table>
<thead>
<tr>
<th>Additions* (final concentration)</th>
<th>% Radioactivity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18:0 18:1 20:1</td>
<td>mmol/min/mg %</td>
</tr>
<tr>
<td>NADH (1 mM)</td>
<td>17 72 12</td>
<td>1.62 ± 0.81 100</td>
</tr>
<tr>
<td>NADH + CO₂</td>
<td>17 72 12</td>
<td>1.53 ± 1.08  94</td>
</tr>
</tbody>
</table>

*The reaction conditions described in Fig. 2 were used.

**TABLE V**

Effect of potassium cyanide on [9-14C]malonyl-CoA incorporation into microsomal fatty acids

<table>
<thead>
<tr>
<th>Additions* (final concentration)</th>
<th>% Radioactivity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2-14C]Malonyl-CoA</td>
<td>16 61 23</td>
<td>2.02</td>
</tr>
<tr>
<td>[2-14C]Malonyl-CoA + KCN</td>
<td>32 43 25</td>
<td>2.00</td>
</tr>
<tr>
<td>(0.5 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2-14C]Malonyl-CoA + KCN</td>
<td>47 32 22</td>
<td>2.11</td>
</tr>
<tr>
<td>(1.0 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[1-14C]Stearoyl-CoA*</td>
<td>60 40 0</td>
<td>4.0</td>
</tr>
<tr>
<td>(0.5 mM)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[1-14C]Stearoyl-CoA + KCN</td>
<td>99 1 0</td>
<td>0</td>
</tr>
<tr>
<td>(1.0 mM)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[1-14C]Stearoyl-CoA + KCN</td>
<td>100 0 0</td>
<td>0</td>
</tr>
<tr>
<td>(0.0 mM)*</td>
<td></td>
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*Additions* (final concentration) [2-14C]Malonyl-CoA was replaced by 20 μM [1-14C]stearoyl-CoA (50,000 cpm).

The biosynthesis of unsaturated fatty acids in mammalian cells occurs primarily by the oxidative desaturation of pre-existing long chain fatty acyl-CoA's (for recent review, see Jeffcoat, Ref. 20). These CoA substrates are derived from either the diet, the cytoplasmatic fatty acid synthetase system, the mitochondrial acetyl CoA-dependent elongation system, or the endoplasmic reticulum malonyl-CoA-dependent elongation system. It is well established that the mono-unsaturated fatty acids are synthesized by a membrane-bound multicomponent system, comprised of NADH-cytochrome b₅ reductase, cytochrome b₅, lipid, and the terminal desaturase complex (21-23). Recently, our laboratory (11) provided evidence suggesting that cytochrome b₅ was also involved in the NADH-dependent microsomal elongation of fatty acids. The present paper describes: (i) additional correlations between the rates of cytochrome b₅ reoxidation and malonyl-CoA incorporation, (ii) the biochemical properties of the intact microsomal elongation system, and (iii) identification of the products of the microsomal elongation reaction.

In our studies, the cytochrome b₅ reoxidation rates paralleled the malonyl-CoA incorporation rates under all conditions examined. As indicated earlier, the malonyl-CoA-enhanced cytochrome b₅ reoxidation rate was always slightly faster (10 to 15%) than the malonyl-CoA incorporation rate. This could be readily accounted for by the presence of other reactions involving cytochrome b₅; for example, during microsomal elongation, the formation of palmitoyl-CoA and stearoyl-CoA would result in the Δ⁹ desaturation of these substrates, since our in vitro assay system would catalyze not only elongation but also desaturation. Hence, both elongation and desaturation would be reflected in the b₅ reoxidation rate.
whereas the malonyl-CoA incorporation assay would measure only elongation. Finally, the apparent $K_m$ values determined for malonyl-CoA by measuring both enhanced cytochrome $b_5$ reoxidation rate and the malonyl-CoA incorporation rate were of the same order of magnitude, 4.7 $\mu$M versus 8.5 $\mu$M. Podack et al. (24) reported a $K_m$ of 22 $\mu$M for the malonyl transfer reaction of the partially purified rat liver microsomal chain elongation system. Bernert and Sprecher (25) found that for the initial condensation reaction the apparent $K_m$ for malonyl-CoA ranged from 47 to 51 $\mu$M with three different acyl-CoA substrates. It is reasonable to state that these apparent $K_m$ values are not significantly different, in view of the fact that the assay conditions employed were radically different. Besides characterization of the biochemical properties of the elongation system, these data show a strong correlation between the rates of $b_5$ reoxidation and incorporation and demonstrate that fatty acid elongation can be monitored by either method.

As indicated earlier by Nugteren (1), optimal microsomal chain elongation occurred with either NADH or NADPH. Similar observations were made in our laboratory when endogenous fatty acids were employed as substrates. However, it was noted that the initial rates of malonyl-CoA incorporation were slightly faster with NADPH, although the percentage radioactivity in each product was essentially the same with either pyridine nucleotide. The apparent $K_m$ for each nucleotide (7 $\mu$M for NADPH, 8 $\mu$M for NADH) indicated little or no preference of the enzyme for either one in the elongation reaction.

In studying the biochemical properties of the microsomal fatty acid chain elongation system, care must be taken in the isolation of the microsomal fraction. As noted in Table I, the two possible major contaminants of isolated microsomes, the cytosolic fatty acid synthetase and the mitochondrial elongase, did not contribute significantly to the elongation activity. The liver microsomal chain elongation of endogenous fatty acids resulted in the formation of an 18:1 fatty acid, with some formation of 18:0 and 20:0 fatty acids. Since our assay was performed under aerobic conditions, both elongation and desaturation occurred simultaneously. As a result, several possible reaction pathways exist, which singly or combined, could give rise to the observed fatty acids. The C18 fatty acids could be synthesized as follows: 1) elongation of endogenous palmitic acid followed by desaturation to oleic acid, 2) desaturation of endogenous palmitic acid to palmitoleic acid followed by elongation to vaccenic acid, and/or 3) elongation of endogenous palmitoleic acid to vaccenic acid.

The C18 fatty acid could be synthesized by: 1) the elongation of oleic or/and vaccenic acids to 11-20:1 or 13-20:1, 2) elongation of stearic acid to 20:0, 3) desaturation of oleic acid and/or vaccenic acid followed by elongation to 20:2, and/or 4) elongation of linoleic acid to 11,14-20:2.

The most important of these reaction pathways (as will be explained below) can be summarized by the following scheme

$$\text{where } E \text{ and } D \text{ represent elongation and desaturation pathways respectively. Contrary to our results are the findings of Landriscina et al. (14) who reported that 23% of the total endogenous fatty acids synthesized was 18:0, 15% was 18:1 (presumed to be oleic acid) and 15% was 16:3. Their results could be explained, in part, by the fact that their assay mixture contained KHCO₃ and acetyl-CoA, in addition to malonyl-CoA, allowing de novo synthesis to occur due to cytosolic enzymes present within the microsomal vesicle.}

As Table III indicates, palmitoyl-CoA and palmitoleoyl-CoA are substrates for the microsomal elongation system. The sum of the specific activities obtained with these two substrates was equal to the activity obtained with the endogenous fatty acids, suggesting that the prime endogenous substrates are palmitic and palmitoleic acids and these two substrates give rise to all the 18:0 and 18:1 formed from the endogenous fatty acids. In support of this conclusion 1.0 mM KCN inhibits partially, but not completely, the formation of 18:1 with a concomitant increase in 18:0.

On the basis of the percentage of radiolabel in the C9 and C11 aldehyde acids following ozonolysis, the 18:1 peak contained 40% vaccenic acid and 60% oleic acid. Furthermore, as seen from Table III, the rates of elongation of palmitoyl-CoA (0.81 nmol/min/mg) and palmitoleoyl-CoA (0.54 nmol/min/mg) suggested such a proportion. The data obtained with KCN, assuming 70% inhibition of $\Delta_5$ desaturation were also compatible with a 40:60 ratio of vaccenic acid to oleic acid.

With regards to inhibition, the KCN experiments produced some unexpected results. For example, 1 mM KCN completely inhibited $\Delta_5$ desaturation of exogenously added stearoyl-CoA (Table V) and palmitoyl-CoA (data not shown). However, 1 mM KCN caused only a 70% inhibition of palmitoyl-CoA (16:0) conversion to 18:1 (72% radioactivity versus 22%) via 18:0. It should be recalled that 40% of the 18:1 formed was derived from endogenous palmitoleic acid which would only undergo elongation. Hence the data suggest that an endogenously generated substrate of the $\Delta_5$ desaturase is not as efficiently inhibited by KCN as an exogenously administered substrate. Why KCN has less ability to inhibit two successive concerted reactions (elongation followed by desaturation) than a direct desaturation of an exogenous substrate is presently not known.

The formation of the radiolabeled C20 fatty acid appears to be primarily derived from oleic acid or linoleic acid for several reasons. First, it is unlikely that the C20 fatty acid could be derived by the elongation of 18:0 to 20:0. Under our assay conditions, stearoyl-CoA was a poor substrate for elongation (Table III). In agreement with our data both Nugteren (4) and Bernert and Sprecher (13) found stearoyl-CoA to be a poor substrate for microsomal chain elongation. Second, the C20 fatty acid is not formed by elongation of vaccenic acid to 13-20:1, as no radiolabeled peak was observed after ozonolysis that could be attributed to a C13 fragment. Finally, the C20 fatty acid could not have been formed by the desaturation of oleic acid followed by elongation as no radioactive peak was observed that could be attributed to any fragment other than C9 and C11. Since the formation of C20 was shown to be uninhibitable by 1 mM KCN, most of the C20 would appear to be primarily synthesized from the endogenously occurring oleic acid or linoleic acid.

Finally, when 100% CO was added to the incubation assay, malonyl-CoA incorporation into endogenous microsomal fatty acids was unaffected (Table IV), suggesting the lack of involvement of the terminal mixed function oxidase, cytochrome P-450 in the microsomal elongation reaction.

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REFERENCES
Liver Microsomal Fatty Acid Elongation