Synthesis of Fatty Acids in Outer and Inner Membranes of Mitochondria*

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SUMMARY

Fatty acids can be synthesized in mitochondrial membranes of rat liver either by elongation or by synthesis de novo. The outer membranes incorporate greater amounts of substrate into fatty acids than do the inner membranes. Outer membranes synthesize 6- and 8-carbon fatty acids completely de novo, while smaller amounts of 10-carbon fatty acids are produced by the mechanism de novo. The reactions de novo utilize acetyl coenzyme A more effectively than malonyl coenzyme A, and synthesis de novo occurs in the absence of exogenous ATP. Elongation, which produces fatty acids 14 carbon atoms long and longer, depends on added ATP, and acetyl-CoA and malonyl-CoA are incorporated about equally. Synthesis de novo of shorter chain fatty acids by outer membranes continues in the presence of ATP and the concomitant elongation reactions. Inner membranes synthesize only longer fatty acids by elongation. If synthesis de novo of fatty acids occurs in the inner membranes, these acids might be removed by oxidation.

The ATP concentrations in these experiments were compared with the levels of ATP produced by actively respiring liver mitochondria. During metabolically adverse conditions within the cell, the capacity for manufacturing fatty acids by elongation may fluctuate with the ATP levels whereas synthesis de novo continues.

Fatty acid biosynthesis occurs by different mechanisms in different parts of the cell. Complete synthesis de novo in the cytoplasm utilizes malonyl coenzyme A formed by acetyl coenzyme A (1-3). Malonyl-CoA is also the substrate for fatty acid elongation by the microsomes (4, 5). Mitochondria carry out elongation (2) with either acetyl coenzyme A or malonyl coenzyme A as a substrate. Several workers (6-13) report that mitochondria synthesize long chain fatty acids by a process de novo. However, it has not always been possible to duplicate this synthesis de novo with whole mitochondria, and some investigators (14-17) have reported that elongation is the only fatty acid synthetic mechanism in mitochondria. The variation in results may be due partly to different techniques of isolating and incubating mitochondria, partly to the tissue source of the mitochondria, and partly also to variations in the analytical methods for determining synthesis de novo and elongation.

The recent development of techniques for separating mitochondria into their membranous components (18-22) prompted me to initiate studies on mitochondrial fatty acid synthesis in membranous preparations in order to establish the location and importance of the two types of fatty acid biosynthesis. I used the methods of Parsons et al. (22), which seemed most advantageous for isolating the mitochondrial membranes.

This study extends earlier work (22) on fatty acid synthesis and its controls in mitochondrial membranes. Without ATP, synthesis de novo occurs in the outer membrane and yields fatty acids up to 8 or 10 carbon atoms long. With ATP, the elongation reaction, which takes place in both the inner and outer membranes, accounts for most of the substrate incorporated and yields long chain saturated and unsaturated fatty acids.

MATERIALS AND METHODS

Materials—[1-14C]Acetyl coenzyme A (45 µCi per µmole) and [1,3-14C]malonyl coenzyme A (9.2 µCi per µmole) were purchased from New England Nuclear. All other reagents were from commercial sources.

Isolation of Subcellular Fractions—Livers from 130- to 160-g male rats (Sprague-Dawley strain) were removed in a cold room at 4°, minced with scissors, washed twice in a medium of 0.32 M sucrose containing 0.1 mM EDTA and 1 mM Tris buffer at pH 7.2, passed through a Fisher Press into 50 ml of the cold stirred medium, and homogenized by three passes in a Teflon Potter-Elvehjem homogenizer in the same medium. The total homogenate of 20 to 30 livers was brought to 15% concentration, and the whole mitochondria were isolated by differential centrifugation as described by Parsons et al. (22). The mitochondria were taken up in 0.02 M potassium phosphate, pH 7.2, containing 0.02% human serum albumin, and the outer and inner membranes were fractionated twice on discontinuous sucrose gradients (22) and resuspended in 0.25 M sucrose. From 150 to 200 g of fresh liver,

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3 to 4 mg of outer mitochondrial membrane protein and about
twice this much pure inner membrane were usually obtained.
Incubation conditions are described in Table I.

Morphological and Enzymatic Identification of Membranes—
Electron microscopy of the nonfixed, negatively stained mito-
chondrial membranes was performed by Dr. W. H. Fahrenbach.
Monoamine oxidase was measured by the method of Tabor,
Tabor, and Rosenthal (25) and succinoxidase by oxygen con-
sumption with succinate as substrate.

Isolation and Identification of Fatty Acids—Incubation was
terminated by the addition of 0.5 ml of 1 M KOH-50% ethanol.
The reaction products were transferred to screw cap tubes with
two 1-ml washes of the KOH-ethanol and heated at 60°C for 1
hour; excess ethanol was blown off with nitrogen and the contents
were diluted with 5 ml of water. Three petroleum ether (b.p.
range 40-60°C) extracts were discarded and the tube contents were
acidified with HCl and extracted four times with 8-ml aliquots of
petroleum ether. The combined extract was washed twice with
water containing 100 μmoles of acetate made to pH 1 with HCl.
Aliquots of the petroleum ether extract were radioassayed, de-
carboxylated (3), or methylated and subjected to gas chromato-
graphy.1 Fatty acids were identified by co-chromatography with
carrier standards. Individual fatty acids were collected in
Pasteur pipettes containing glass wool wetted with scintillation
fluid. After the pipettes had been rinsed out with scintillation
fluid and the fatty acids radioassayed, some of the acids were
extracted from the scintillation fluid (26) and decarboxylated.

The heptylamine from the decarboxylation of caprylic acid
(8:0)2 was converted to the p-toluenesulfonyl derivative for
further analysis (27).

The decarboxylation ratio indicates the type of synthesis. If
elongation is the only reaction, then the radioactivity located
exclusively in the carboxyl carbon of the fatty acids becomes the
radioactivity of the carbon dioxide that is cleaved by decar-
boxylation. The ratio of total fatty acid radioactivity to car-
boxyl radioactivity would then be 1. Synthesis de novo dis-
tributes the radioactivity throughout the fatty acid molecules
so that decarboxylation yields only a fraction of the total radio-
activity as carbon dioxide. The ratio of total to carboxyl radio-
avity is greater than 1 and would be, e.g. 5 for capric acid
(10:0 equivalent to 5 acetyl units).

The term "synthesis de novo" is applied here to any fatty acid,
regardless of length, in which the decarboxylation ratio indicates
that exogenous radioactive acetyl units were incorporated into
the carboxyl and the terminal methyl carbons, as well as into the
other chain carbons. Elongation is the addition of a radioactive
acetyl unit to the carboxyl end of a nonradioactive endogenous
fatty acid. Estimates of the percentage of synthesis de novo
were calculated by the formula

\[
\% \text{ synthesis de novo } = 100 \left( \frac{DR - 1}{CL - 1} \right)
\]

1 Gas chromatography was carried out on a Barber-Colman
 instrument containing a glass U-tube, 6 feet × 6 mm, internal
diameter, packed with 15% Hi-Eff 2 BP (ethylene glycol succinate)
on Gas-chrom P, 80 to 100 mesh (Applied Science Laboratories,
College Park, Pennsylvania). Temperature was initially 110°C
and was raised to 190°C after the emergence of C 8:0 at 4 min.
Carrier N₂ flowed at about 200 ml per min.

2 Fatty acid designation is the following: the number preceding
the colon is the number of carbon atoms in the chain; the number
following the colon is the number of double bonds.
in which DR is the decarboxylation ratio and CL is the length of
the fatty acid in acetyl units, i.e. the number of carbon atoms
divided by 2.

RESULTS

Characterization of Mitochondrial Membranes—Electron micro-
graphs showed that the outer membranes were of the same gen-
eral appearance and size as the vesicular structures pictured by
Parsons et al. (21). With the isolation procedure used, there
was no visible contamination of the outer by the inner mem-
brane, and only a very few (<2%) smaller vesicles, either
microsomes or fragmented mitochondrial membranes, were pres-
ent. The inner membranes were identified by the 90° project-
ing subunits and were free of membranous components that did
not have the projections. Succinoxidase was present almost ex-
clusively in the inner membrane.

Outer membranes had the highest monoamine oxidase specific
activity (138 μmoles of benzylamine oxidized per mg of protein
per min), which was much greater than the activity in the inner
membranes (10 μmoles per mg per min). Schnaitman, Erwin,
and Greenawalt (28), with a slightly different isolation techni-
que, established that this enzyme is present in the mitochondrial
outer membrane (208 μmoles per mg of protein per min), and
later (29) verified the validity of this assay (25) for use with
outer membrane preparations. Tipton (30), by using the initial
steps of Parsons et al. (21), also found a high concentration of
monoamine oxidase present in the outer membranes.

Synthesis of Fatty Acids—Fatty acid synthesis by the mito-
chondrial preparation is shown in Table I. Both reduced pyri-
dine nucleotides were included for maximum substrate incorpo-
ration by the outer membrane; with only one present synthesis
was reduced 50 to 60%. The outer membranes incorporated the
greatest amount of substrate into fatty acids, the whole mito-
chondria incorporated least. The amount of substrate incorpo-
rated by the inner membrane was comparable to that incorpo-
rated by whole mitochondria but could be increased by adding
ATP. Presumably, a finite amount of fatty acids, and sub-
strate, would be oxidized by the whole mitochondria and the
inner membrane so that the final results represent a net sum of
the anabolic and catabolic reactions. Whether fatty acids are
also lost through oxidation in the outer membranes remains to
be established.

Outer membranes incorporated 8 to 10 times more acetyl-CoA
than any other fraction either with or without ATP. In the ex-
periments listed, acetyl-CoA was incorporated about twice as
effectively as malonyl-CoA without ATP; this difference ranged
as high as 4-fold in other runs. When ATP was present, the
incorporations were more nearly equal. Because the large
amount of acetyl-CoA incorporated by outer membranes without
ATP was most interesting, this fraction was examined further.

Decarboxylations (3) were performed on aliquots of the total
fatty acid extracts to determine the type of synthesis with and
without ATP. In the early stages of this work, the decarboxyla-
tion ratios of different preparations ranged from 3.1 to 7.7 (av-
average, 5.2) without ATP. In order that it be established exactly
which individual fatty acids had been synthesized, the petroleum
ether extracts were methylated and the acids were separated by
gas chromatography.

The forerun of fatty acids less than 12 carbon atoms long was
collected in one fraction and the individual fatty acids 12 carbon
atoms and longer were measured separately. However, the fort-
Fatty Acid Synthesis in Mitochondrial Membranes

Table I
Incorporation of [1-14C]acetyl-CoA or [1,3,14C]malonyl-CoA into fatty acids of rat liver mitochondrial fractions and their decarboxylation ratios

The tubes contained 100 mM potassium phosphate, pH 7.2; 6 mM glutathione; 80 μM [1-14C]acetyl-CoA or 81 μM[1,3-14C]malonyl-CoA; 1 mM NADH; 1 mM NADPH; and 0.5 to 1.0 mg of protein. The final volume was 0.5 ml and the capped tubes were incubated at 37° for 30 min with shaking. MgCl₂ and MnCl₂ were present with ATP in the ratio of ATP-Mg-Mn = 1:0.4:0.4. Protein was measured by the method of Lowry et al. (24).

<table>
<thead>
<tr>
<th>Tissue fraction and additions</th>
<th>[1-14C]Acetyl-CoA</th>
<th>[1,3-14C]Malonyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Decarboxylation ratio</td>
</tr>
<tr>
<td>Whole mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>19, 43</td>
<td>1.5</td>
</tr>
<tr>
<td>0.01 mM ATP</td>
<td>19, 56</td>
<td>1.3</td>
</tr>
<tr>
<td>0.1 mM ATP</td>
<td>30, 62</td>
<td>1.2</td>
</tr>
<tr>
<td>Mitochondrial outer membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1174, 1318</td>
<td>3.2</td>
</tr>
<tr>
<td>0.01 mM ATP</td>
<td>1070, 2066</td>
<td>2.5</td>
</tr>
<tr>
<td>0.1 mM ATP</td>
<td>2059, 1794</td>
<td>1.4</td>
</tr>
<tr>
<td>Mitochondrial inner membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>25, 54</td>
<td>1.0</td>
</tr>
<tr>
<td>0.01 mM ATP</td>
<td>41, 109</td>
<td>1.2</td>
</tr>
<tr>
<td>0.1 mM ATP</td>
<td>124, 351</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Specific activity is measured as μmoles of substrate incorporated per mg of protein per hour. Two separate runs are listed for comparison.

The decarboxylation ratio is given for second run listed.

Table II
Fatty acids synthesized from [1-14C]acetyl-CoA by mitochondrial membranes

Incubation conditions are given in Table I.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Outer membranes</th>
<th>Inner membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Substrate incorporation</td>
</tr>
<tr>
<td></td>
<td>[1-14C]acetyl-CoA</td>
<td>[1,3-14C]malonyl-CoA</td>
</tr>
<tr>
<td>No ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 mM ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:0</td>
<td>62.5</td>
<td>44.1</td>
</tr>
<tr>
<td>8:0</td>
<td>22.1</td>
<td>18.1</td>
</tr>
<tr>
<td>10:0</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>14:0</td>
<td>0.2</td>
<td>2.6</td>
</tr>
<tr>
<td>&gt;14:1 &lt; 16:0</td>
<td>4.5</td>
<td>2.6</td>
</tr>
<tr>
<td>16:0</td>
<td>0.4</td>
<td>2.5</td>
</tr>
<tr>
<td>18:0</td>
<td>0.3</td>
<td>1.6</td>
</tr>
<tr>
<td>18:1</td>
<td>0.4</td>
<td>3.4</td>
</tr>
<tr>
<td>18:2</td>
<td>0.2</td>
<td>1.7</td>
</tr>
<tr>
<td>&gt;18:3 &lt; 22:0</td>
<td>0.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

**Carrier fatty acids were present and fatty acid identification is based on these. Some collection points contained no recordable fatty acids and are designated by those recorded before and after that point.**

*Percentage of the total radioactivity collected from the gas chromatograph excluding the forerun before 6:0 and collecting through 21:1.

Without ATP, the inner membranes incorporated very little radioactivity into fatty acids less than 14 carbon atoms long. With ATP, some radioactivity appeared at 10:0 but most of it was at 12:0 and longer. The percentage of each fatty acid produced at different concentrations of ATP did not show the great individual variations seen in the fatty acids from outer membranes. Also, there was a greater percentage of radioactivity distributed into long chain fatty acids not listed in Table II. Extraction and decarboxylation of the fatty acids synthesized by the inner membrane revealed elongation as the only mechanism for substrate incorporation.

Similar results were obtained from several experiments with outer membranes, i.e. without ATP, 6:0 and 8:0 accounted for most of the substrate incorporation, with very little being incorporated into longer fatty acids. Presumably, though not measured directly, 4:0 accounted for the radioactivity in the forerun since radioactive substrate acetate was not carried through to gas chromatography. From the earlier decarboxylation ratios, it would be expected that little radioactivity would be in the short chain acids and more would be in the longer fatty acids.

The method for obtaining these ratios was re-examined. Since one aliquot had been dried for decarboxylation, and another aliquot had had scintillation fluid added to it to determine total fatty acid radioactivity, the loss of short acids during sample drying might account for higher decarboxylation ratios. To test this possibility, we placed replicate samples of petroleum ether extracts of the total fatty acids synthesized by outer membranes without ATP in vials. Scintillation fluid was added to some without drying; others were dried for various periods of time. Between 32 and 40% of the radioactivity was lost just by taking the sample to dryness under nitrogen and 64 to 81% was lost.
by drying the sample for 10 min. Since the method and duration of drying samples had not been controlled in previous work, the decarboxylation ratios obtained for outer membranes might not have been as accurate for interpreting the type of synthesis and the length of fatty acids synthesized as initially supposed.

So that the loss of volatile fatty acids might be prevented, the extracted acids were examined as their salts. Either aliquots of the alkaline water extract were used or the free acids in petroleum ether were converted to their salts by the addition of ethanolic KOH. When replicates of the latter were exposed to a stream of nitrogen for up to 10 min, virtually no radioactivity was lost. To ensure further against loss, we placed the sample flasks in ice water while they were being dried and added the decarboxylation reagents immediately after the sample was dry.

Decarboxylation ratios for the various fractions are given in Table I. Outer membranes, without added ATP and with acetyl-CoA as substrate, now reproducibly gave ratios of about 3 as would be expected for predominant synthesis de novo of short chain fatty acids. Whole mitochondria gave ratios close to 1 when acetyl-CoA was used as the substrate so that apparently elongation was the major reaction. Malonyl-CoA yielded higher ratios with whole mitochondria, a probable indication that elongation was not the only synthetic reaction taking place. These ratios were close to those obtained with outer membranes; but because the total activity of the fatty acids synthesized was small, no analysis of individual fatty acids could be made. The inner membrane reaction was also one of elongation when acetyl-CoA was present and again the ratio was slightly higher for malonyl-CoA.

Malonyl-CoA plus outer membranes gave low decarboxylation ratios, an indication primarily of elongation, even without ATP. However, acetyl-CoA incubated with outer membranes resulted in ratios of slightly over 3 when ATP was not present. This could mean synthesis de novo of a 6-carbon fatty acid or some composite figure resulting from the varied type of synthesis and chain length produced. The major distribution of radioactivity into short chain fatty acids, as shown in Table II, taken with the decarboxylation ratio, indicated the probability of synthesis de novo for these acids. As ATP was added, the decrease in the decarboxylation ratio for aliquots of the total extract was probably due to an increase in the elongation reactions so that the decarboxylation ratios of 3 or 4 from short chain fatty acids synthesized de novo was averaged with the ratio of 1 from the increasing amount of elongated acids. This reduced the composite average for the total fatty acids. Since the radioactivity incorporated into the longer chain fatty acids increased when ATP was added and the decarboxylation ratio decreased, it appears that the increased substrate incorporation was mainly via elongation. The effect of ATP on substrate incorporation and decarboxylation ratios is shown graphically in Fig. 1. Increasing ATP caused greater substrate incorporation, but this was due almost exclusively to increased elongation, as shown by the decrease in decarboxylation ratios to values approaching 1. So that the type of synthesis for specific fatty acids could be identified, certain acids from the different outer membrane incubations were decarboxylated.

Decarboxylation ratios for various fatty acids from outer membrane preparations are shown in Table III. The most noticeable feature is that the 6- and 8-carbon fatty acids were assembled completely from radioactive acetyl units either with or without the addition of ATP. The decarboxylation ratio for the 10-carbon fatty acid indicates that some 40% of that acid could have been synthesized de novo. The greatest incorporation of radioactivity into fatty acids 14 carbon atoms long and the longer chain fatty acids was in the presence of ATP, and the acids were formed by elongation. This is similar to the fatty acids produced by the inner membrane in that the major acids are long chain and radioactivity is incorporated by elongation.

Two metabolites, acetocetate acid and β-hydroxybutyric acid, were considered as possible contaminants since they can be produced by the liver mitochondria. If these compounds were carried through extraction to gas chromatography, the keto or hydroxyl function could conceivably have delayed the 4-carbon acid so that it emerged at the same time as 6:0 or 8:0. Methylation and gas chromatography of known samples showed that these two metabolites emerged after 10:0 and thus were excluded as contaminants of 6:0 and 8:0.

The techniques of isotope dilution were used to characterize caprylic acid (8:0). After decarboxylation of the capryl acid synthesized de novo, the resultant heptylamine should still contain radioactivity. The flask contents were made basic with NaOH, the radioactive heptylamine was extracted, and non-radioactive heptylamine was added. The p-toluenesulfonyl derivative of the heptylamine was prepared (27) and recrystallized.

![Figure 1: Effect of varied ATP concentration on fatty acid synthesis in outer membranes of mitochondria.](image-url)
to constant specific activity, within experimental error. If the radioactivity had been other than [14C]heptylamine arising from [14C]caprylic acid, the derivative should have lost radioactivity and lowered the specific activity with each recrystallization. As a further check, [14C]pontamylamine was extracted after deacetylilation of [14C]caproic acid (6:0) and diluted with nonradioactive heptylamine, and the p-toluenesulfonyl derivative was prepared. The decrease in specific activity with each recrystallization indicated the loss of the radioactive compound and the specificity of this technique.

The data in Table II show a decline in the percentage of acetyl-CoA incorporated into the shorter fatty acids when ATP is added and more elongation of longer chain fatty acids. To check this, we made approximations of the amount of substrate incorporated into a few of the fatty acids by multiplying the total activity of the fraction by the percentage activity of each fatty acid; the results are listed in Table IV. The increase in total activity, which was due to greater amounts of substrate incorporated into longer chain fatty acids, makes it appear that the shorter chain fatty acids contribute a smaller percentage of the total activity. Actually, the synthesis de novo of short chain fatty acids remained relatively constant, within the accuracy of these calculations, whereas the elongation of long chain fatty acids, dependent on ATP, increased. This seems to indicate that the two enzymatic systems operate separately. Their interdependence, if any, remains to be established.

**DISCUSSION**

Fatty acids can be synthesized either by utilizing 2-carbon acetyl units to build them completely de novo or by elongating pre-existing fatty acids with an acetyl unit. This work establishes that a system that synthesizes de novo short chain fatty acids is present in the outer membranes of mitochondria. Enzymatic reactions for elongating longer chain fatty acids are present in both outer and inner membranes, and this system is directly dependent upon ATP.

It is clear that mitochondrial outer membranes can synthesize caproic (6:0) and caprylic (8:0) acids de novo. This is evident from their decarboxylation ratios approaching the theoretical values of 3 and 4, respectively. Caproic acid is the major fatty acid synthesized and the amount of caprylic acid is about one-third that of caproic acid. Butyric acid (4:0), though not specifically sought, would have been present in the solvent front. The decarboxylation ratio of 2.9 for capric acid (10:0) without ATP can be interpreted as an average ratio for about 40% synthesis de novo and 60% elongation. Alternatively, endogenous butyric acid, if present, could have been elongated by 3 acetyl units to give that ratio. This does not seem as likely, since elongation with these preparations involved the addition of only 1 acetyl unit, at least for the longer chain fatty acids. The decarboxylation ratios for myristic (14:0) acid and longer acids (16:0 to 20:0) showed elongation by only 1 acetyl unit in both outer and inner membranes. As measured under these conditions, the synthesis de novo of short chain fatty acids is carried out almost exclusively in the outer membrane. Even if these acids were synthesized in the whole mitochondria or inner membrane, oxidation could prevent their accumulation and thereby account for the low activity of these preparations. Malonyl-CoA may serve as a substrate for synthesis de novo after decarboxylation (33, 34) to acetyl-CoA. The reverse is less likely since acetyl-CoA is carboxylated relatively slowly by propionyl-CoA carboxylase (35). Also, the carboxylation of acetyl-CoA requires ATP (36) and the synthesis de novo of short chain fatty acids is independent of exogenous ATP.

The short chain fatty acids may be synthesized by an enzyme system that is specific for that function, or they may be produced by other enzymes that usually function in a different capacity. Chief among these latter possibilities is synthesis by reversal of the fatty acid oxidation enzymes. A number of reports (37-39) have established the feasibility of synthesizing short chain fatty acids by the use of the enzymes of β oxidation. The results of our work fit in well with the proposal that synthesis de novo of short chain fatty acids could occur by reversal of the fatty acid oxidation enzymes. However, data on the intramitochondrial localization of the fatty acid oxidation enzymes are somewhat equivocal. Two reports on the direct measurement of fatty acid oxidation in mitochondrial membranes state opposite conclusions. Allman et al. (40) placed the oxidizing enzymes in the outer membranes, but Beattie (41), with an identical assay but different isolation techniques (28), concluded that the enzymes are in the inner membrane. Waite (42) has pointed out that the phospholipase treatment used by Allman et al. (40, 48) causes extensive cross-contamination of membrane components and enzymes in the different fractions. Evidence from other workers (43-45) supports the localization of the β oxidation enzymes in the inner membrane or at least farther inside the mitochondria than the outer membrane surface. Even though the hypothesis that short chain fatty acid synthesis in the outer membranes can occur by reversal of β oxidation is plausible, most evidence indicates that the β oxidation enzymes are not associated with the outer membranes. It may be that a specific discrete enzyme system exists in the outer membrane that synthesizes short chain fatty acids as its primary function, or there may be other less specific enzymes that synthesize short chain acids.

The establishment of the elongation of fatty acids by mitochondria supports similar observations by numerous workers (5, 14-17, 38, 46). The current data supply no additional insight as to whether malonyl-CoA or acetyl-CoA is the immediate substrate utilized for elongation. An additional complicating factor is that carboxylating and decarboxylating enzymes are spatially separated in the mitochondria (34) and cell, and this may determine the substrate utilized in elongation. Presumably ATP participates in the production of acyl-CoA compounds, assuming that in the preparations there is a small residual amount of coenzyme A or that coenzyme A is generated from the radioactive substrate supplied. There is evidence that the acyl-CoA synthetase enzyme, which, with ATP, activates long chain fatty

### Table IV

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0.01 mm ATP</th>
<th>0.1 mm ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ATP</td>
<td>1318</td>
<td>2066</td>
<td>3570</td>
</tr>
<tr>
<td>6:0</td>
<td>822</td>
<td>915</td>
<td>740</td>
</tr>
<tr>
<td>8:0</td>
<td>291</td>
<td>375</td>
<td>430</td>
</tr>
<tr>
<td>10:0</td>
<td>0</td>
<td>89</td>
<td>456</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity is the percentage of radioactivity collected by gas chromatography times the total activity of the sample.

**TABLE IV**

Approximation of absolute amounts of fatty acids synthesized by outer membranes with [1-14C]acetyl-CoA

**Fatty Acid Synthesis in Mitochondrial Membranes**

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acids to their coenzyme A derivatives, is present predominantly in the outer membrane of mitochondria (43, 47, 48).

At an ATP concentration of 0.01 mM, elongation by the outer membranes accounted for about 20 to 25% of the substrate incorporated. In terms of the actual amounts of radioactive fatty acids isolated from the two enzymatic reactions, elongation yielded about 40% of these acids, and this percentage was even greater at higher ATP concentrations. Ernster et al. (49) found that the ATP concentration in actively respiring rat liver mitochondria was about 7 mM. ATP per mg of protein. The 0.01 mM ATP used here was equivalent to about 15 mM per mg of protein and, at this concentration, elongation actively proceeded. At higher ATP concentrations, i.e. 0.1 mM, elongation was even more pronounced; this ATP level, and the higher levels (4 to 10 mM) used by other workers (14-17), may be far above the amount of ATP actually produced by mitochondria in vivo. Under adverse conditions in vivo, a decrease in ATP availability would decrease elongation, but synthesis de novo might still produce fatty acids for, as yet, unknown uses.

The results presented here confirm other observations that stearic acid (18:0) and longer saturated and unsaturated fatty acids are synthesized primarily by elongation. This is agreed upon by all workers, including those who have reported on the synthesizing of de novo capabilities of mitochondria (9, 11-13). The predominance of longer chain fatty acids arising by elongation probably reflects the greater amounts of medium and long chain fatty acids endogenously available to serve as substrates for the reaction.

Whether laurie (12:0), myristic (14:0), and palmitic (16:0) acids can be synthesized de novo in mitochondria is still debatable. Whether laurie, myristic, and some palmitic acid was not ruled out by synthesis results. However, the possibility of synthesis of laurie and myristic acids de novo is the only fatty acid-synthesizing system in mitochondria, and the probability that the coenzyme A derivatives, is present predominantly in the outer membrane of mitochondria (43, 47, 48).

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REFERENCES


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