FORMATION OF ACETOACETATE FROM FATTY ACIDS BY PARTICULATE SYSTEMS OF RAT LIVER*

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The relative importance of oxidation through the Krebs cycle or of conversion to acetoacetate for the oxidation of fatty acids in liver is unknown. Attempts have been made to answer this question by studies of the products of the oxidation of fatty acids in particulate systems (1-7). In most of the experiments this process has required the presence not only of adenine nucleotides, magnesium ion, and cytochrome c, but also "activators" such as heated extracts of liver (6), intermediates of the Krebs cycle (2, 4, 7), or reduced diphosphopyridine nucleotide (DPN) (3). The type of activator added apparently determines the pathway of oxidation (2, 3). Thus in the presence of reduced DPN the carbon chains of both long and short chain acids are converted to acetoacetate (3), whereas, when sources of oxalacetate are added, the fatty acids, particularly those with long chains (2), have a greater tendency to be oxidized through the Krebs cycle (2, 7, 8). However, little information is available as to the pathway of oxidation of fatty acids in systems which do not require the addition of such activators (1, 5). Since these undoubtedly contain intact activating systems, it seems possible that a closer approximation to the major pathway of fatty acid oxidation in liver might be obtained by further study of such preparations.

Therefore, the effect of chain length of the fatty acid on acetoacetate yield was investigated in systems which did not require the addition of activators for maximal rate of oxidation. The fatty acids studied were the homologous series of fatty acids ranging in chain length from C₂ to C₁₇. The particulate systems used included washed particles from liver, liver mitochondria prepared in hypertonic sucrose, and liver mitochondria prepared in isotonic sucrose. The results obtained emphasize the importance of conversion to acetoacetate in the oxidation by liver of fatty acids of even carbon number.

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FORMATION OF ACETOACETATE

EXPERIMENTAL

Most of the experimental details are given in previous publications (6, 9). Particles from rat liver were washed three times with isotonic potassium chloride (6); mitochondria were prepared in hypertonic sucrose by the precipitation method of Kennedy and Lehninger (10) and washed three times with isotonic potassium chloride. Mitochondria were prepared in isotonic sucrose by a modification of the method of Kielley and Kielley (11) and washed two times with isotonic sucrose. A homogenizer of the type described by Dounce and Beyer (12) was used. The "fluffy" layer was removed from the sedimented mitochondria, which were always kept at 0°.

The fatty acids used in this study were obtained from the Eastman Kodak Company and when necessary were subjected to further purification. The sources of the other materials used in these experiments have been given in previous publications from this laboratory (6, 9). Acetoacetate was estimated as acetone by the sensitive vanillin method (13). Occasionally these values were checked by the aniline citrate method (14). Adenosinetriphosphatase (ATPase) was measured by the method of Dubois and Potter (15). The rate of oxygen uptake was measured by the usual Warburg manometric technique. The substrate was added to the ice-cold reaction mixture, and the flasks were incubated in the bath at 30° for 5 minutes before the stop-corks were closed. The fatty acids were neutralized with sodium hydroxide, and those of chain length greater than 10 carbons were added as fresh hot (90°) solutions to the chilled Warburg flask. Except where noted, air was the gas phase.

The reaction mixture for the studies with the washed particles or with mitochondria prepared in hypertonic sucrose contained 0.015 M tris(hydroxymethyl)aminomethane, pH 8.0, 0.01 M potassium phosphate, pH 8.0, 3 × 10⁻⁶ M cytochrome c, 0.0066 M magnesium chloride, 0.05 M potassium chloride (this includes the salt in the enzyme suspension), 0.0013 M adenosinetriphosphate (ATP), and 0.001 to 0.00025 M fatty acids, particulate suspension equivalent to 300 mg. of fresh liver, and water to make a total volume of 3.0 ml. The reaction mixture used with the isotonic mitochondria was similar except for the presence of 0.002 M ATP, 0.05 M sucrose, 0.03 M potassium chloride, 0.01 M creatine, and 10 mg. of a dialyzed fraction of rabbit muscle precipitating between 50 and 70 per cent saturation with ammonium sulfate (16).

Results

In Table I are presented the results of studies of the effect of chain length of the acid on the formation of acetoacetate with mitochondria prepared in isotonic sucrose or with washed particles as the source of the
fatty acid oxidase. Similar results were obtained with representative acids when mitochondria prepared in hypertonic sucrose were used, but for purposes of brevity these have been omitted. The experimental values have been corrected for the small amount of respiration and acetoacetate formation which took place in the absence of added substrate. Also by extrapolation of the rate curves, the total oxygen uptake has been corrected for the first 5 minutes the flasks were in the bath with the stoppers open. The oxidation was allowed to go to completion with the acids of even carbon number, but was stopped after the rate had become very slow with the acids of odd carbon number. In the case of the acids of even carbon number the theoretical values for acetoacetate formed and oxygen consumed are calculated for the conversion of the entire carbon chain to acetoacetate. With the acids of odd carbon number these values are calculated for the formation of 1 mole of acetoacetate per mole of fatty acid oxidized, except for propionic acid which is assumed to yield 2 of its carbons for the synthesis of acetoacetate by a recondensation mechanism.

In Table I are recorded the actual experimental data obtained for the

<table>
<thead>
<tr>
<th>Acid added</th>
<th>Mitochondria from isotonic sucrose</th>
<th>Washed particles</th>
<th>Theoretical values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of carbon</td>
<td>Amount</td>
<td>Rate of oxygen uptake</td>
<td>Total oxygen uptake</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>1.8</td>
<td>3.2</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>3.0</td>
<td>6.2</td>
</tr>
<tr>
<td>7</td>
<td>3.0</td>
<td>3.2</td>
<td>12.5</td>
</tr>
<tr>
<td>8</td>
<td>3.0</td>
<td>3.8</td>
<td>9.0</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>3.9</td>
<td>17.6</td>
</tr>
<tr>
<td>10</td>
<td>3.0</td>
<td>4.0</td>
<td>6.1</td>
</tr>
<tr>
<td>11</td>
<td>0.75</td>
<td>3.2</td>
<td>6.8</td>
</tr>
<tr>
<td>12</td>
<td>0.75</td>
<td>2.6</td>
<td>3.7</td>
</tr>
<tr>
<td>13</td>
<td>0.75</td>
<td>2.9</td>
<td>5.3</td>
</tr>
<tr>
<td>14</td>
<td>0.75</td>
<td>2.0</td>
<td>4.6</td>
</tr>
<tr>
<td>15</td>
<td>0.75</td>
<td>2.0</td>
<td>5.6</td>
</tr>
<tr>
<td>16</td>
<td>0.75</td>
<td>1.5</td>
<td>4.2</td>
</tr>
<tr>
<td>17</td>
<td>0.75</td>
<td>1.5</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Oxygen uptake per 10 minutes per equivalent of 300 mg. of wet liver.
rates of oxidation, acetoacetate formed, and the total oxygen consumed. These values are not always strictly comparable from one acid to another, because all of the acids were not studied at the same concentration. However, since concentration units are canceled in the calculation of the acetoacetate to oxygen ratios, the latter more clearly reflect differences in mode of oxidation between the acids.

The results in Table I show that the complete carbon chain of acids with an even number of carbons ranging from C₄ to C₁₆ were converted quantitatively to acetoacetate. The quantitative formation of acetoacetate from acids such as C₆, C₁₀, or C₁₄ certainly supports the results of isotope studies (17), which indicate a recondensation mechanism for the formation of acetoacetate by rat liver. The striking decrease in the acetoacetate to oxygen ratios which is observed when an acid of odd carbon number is compared with one of even carbon number shows that the former acids are oxidized with the production of much less acetoacetate than are the latter acids. However, the ratios also indicate that, with the exception of the C₅ and C₇ acids, which gave low ratios, and the C₃ acid, which was not oxidized, the acids with an odd number of carbon atoms yielded more than 1 mole of acetoacetate per mole of fatty acid oxidized. Thus in these preparations the factor which determines whether a fatty acid will be oxidized to acetoacetate is not the chain length, as with mitochondria activated with oxalacetate (1), but whether the number of carbons is odd or even.

These results are in agreement with the many observations (1, 2, 4, 7, 18) that less acetoacetate is produced from acids with an odd number of carbon atoms than from those with an even number of carbon atoms. They confirm and extend the early experiments of Lehninger (1) with the oxidation of octanoate and palmitate by washed particles to which no activator was added, but are in disagreement with similar studies conducted by Weinhouse et al. (5).

In Table I the rate of oxidation of the fatty acids is given for the first 10 minutes and is expressed as micromoles of oxygen per equivalent of 300 mg. of wet liver. It can be seen that the acids from C₄ to C₁₇ were oxidized at good rates by mitochondria prepared in isotonic sucrose, whereas acetic acid and propionic acids were not oxidized. This failure to oxidize acetic and propionic acids has been noted with many types of rat liver preparations (1, 7). Similar rates were obtained with washed particles, but the fatty acids were oxidized at about two-thirds these rates by mitochondria prepared in hypertonic sucrose. Schneider (19) previously had noted that the latter preparation gave lower rates of fatty acid oxidation.

The fatty acid oxidase of mitochondria prepared in isotonic sucrose was much more stable than that of the other preparations. This enzyme sys-
tem, when present in washed particles or mitochondria prepared in hypertonic sucrose, is completely inactivated by freezing (20)\(^1\) or by the presence of ferricyanide.\(^2\) However, in Table II it is shown that ferricyanide can be used as an electron acceptor for the oxidation of fatty acids by mitochondria isolated in isotonic sucrose. Also such preparations retain appreciable fatty acid oxidase activity, with oxygen as the electron acceptor, after freezing and storage at \(-77^\circ\). All attempts to prevent the initial drop in activity which occurs on freezing were unsuccessful. The methods tested included the addition of various activators and cosubstrates to the reaction mixture and freezing of the mitochondria in the presence of various protective agents such as gum arabic (21), cysteine, or magnesium chloride (22). The results of centrifugation at 10,000 \(\times\) \(g\) and microscopic examination indicate that many of the mitochondria were broken by the process of freezing and thawing. It is possible that the fatty acid oxidase activity is retained only by the intact mitochondria.

In confirmation of previous results with washed particles prepared in salt solution (8) or mitochondria prepared in isotonic sucrose (23), in none of the preparations used in Table I was the addition of activators necessary for the maximal rate of oxidation of the fatty acids. However, as described by others (23), it was necessary to add a phosphate acceptor, in this case the phosphocreatine system, to the mitochondria prepared in isotonic sucrose in order to permit the maximal rate of oxidation of fatty acids.

\(^1\) Witter, R. F., unpublished observation.

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### Table II

**Oxidation of Hexanoate by Mitochondria**

The oxidation of hexanoate was carried out as described in the experimental section except that the pH was 7.4. In the ferricyanide experiments an atmosphere of 95 per cent nitrogen-5 per cent carbon dioxide replaced the air, and 0.01 mM ferricyanide and 0.015 M sodium bicarbonate were also present.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Oxidant</th>
<th>Treatment of mitochondria</th>
<th>Oxygen uptake(^{#})</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Air</td>
<td>None</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>Ferricyanide</td>
<td>&quot;</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>Air</td>
<td>Frozen at (-77^\circ)</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>Ferricyanide</td>
<td>&quot; &quot; (-77^\circ) and stored at (-77^\circ) for 4 hrs.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>&quot; &quot; (-77^\circ) and stored at (-77^\circ) for 4 hrs.</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>&quot; &quot; &quot; &quot; (-77^\circ) &quot; &quot; (-77^\circ) &quot; &quot; (-15^\circ) for 24 hrs.</td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>&quot; &quot; at (-77^\circ) and stored at (-15^\circ) for 24 hrs.</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{\#}\) Oxygen uptake for 10 minutes per equivalent of 300 mg. of wet liver.
acids and Krebs cycle intermediates. In confirmation of previous observations (11, 23, 24), this mitochondria preparation had very low ATPase activity. The latter was increased on freezing, and concomitantly the addition of the phosphate acceptor was no longer required. These results confirm the generally accepted conclusion that substrates cannot be oxidized in these mitochondria unless some method is provided for the turnover of the phosphate bonds of the ATP formed during the oxidation (23, 24). In contrast to the results of Kennedy and Lehninger (2, 3), the mitochondria prepared in hypertonic sucrose also did not require the addition of activators. These authors (2, 3) pointed out the difficulty of consistently obtaining such preparations and found that best results were obtained by alternately mashing with hypertonic sucrose and isotonic salt solution (3). Since the preparations used in the present study were washed with isotonic potassium chloride, it is possible that the difference in washing procedure may account for the discrepancy in the requirement for activators.

DISCUSSION

In the in vitro systems studied the carbon chains of acids of even carbon number, with the exception of acetic acid, were quantitatively converted to acetoacetate, whereas little of the ketone body was formed from the acids with an odd number of carbons. It is difficult to assess the significance in vivo of these findings, since generalizations based solely on experiments in vitro are subject to criticism. However, these results agree with the well known fact that in vivo the acids with long carbon chains and an even number of carbon atoms yield much more acetoacetate than those with an odd number of carbons (25, 26). They support the hypothesis that this is due to a difference in pathway of oxidation in the liver. However, the results obtained are in apparent disagreement with the observation that the short chain acids are more ketogenic than the long chain acids of even carbon number (27). Since this is apparently not due to differences in rate of absorption (27), it has been attributed to the fact that the short chain acids are not stored (28, 29) and hence must be oxidized (27). Also, the former acids are transported via the portal circulation, whereas the latter are carried via the lymphatic system (30). Accordingly, the short chain fatty acids probably reach the liver in a shorter time than the long chain acids and thus might yield ketone bodies at a faster rate.

Another possible explanation for the ketogenic effect of the short chain acids is a difference in mode of oxidation of the long chain acids by the liver. This hypothesis is not supported by the results presented in this communication. Previous experiments in vitro have shown the short chain acids to be more ketogenic than the long chain acids when oxalacetate (2)
was the activator, but not when DPN was the activator (3). In the present study each of three types of mitochondrial preparations contained an activating system which formed acetoacetate. Since these were made in different media, it seems highly improbable that all three would have the same activating system unless the latter were important in the intact liver. Thus these considerations lend support to the classical concept that formation of acetoacetate is an important pathway of oxidation of naturally occurring fatty acids in liver. However, none of these studies in vitro can be extrapolated to conditions in vivo until the balance of activating systems in the intact liver is known.

**SUMMARY**

The effect of chain length on the acetoacetate yield from each of a series of fatty acids ranging in chain length from C₂ to C₁₇ was investigated with particulate preparations from rat liver which did not require the addition of activators for the maximal rate of oxidation. The systems studied included washed particles, mitochondria prepared in isotonic sucrose, and mitochondria prepared in hypertonic sucrose. With the exception of acetic and propionic acids, the acids were oxidized at vigorous rates. The carbon chains of acids with an even number of carbons, with the exception of acetic acid, were converted quantitatively to acetoacetate, whereas those with an odd number of carbons yielded slightly more than 1 mole of acetoacetate per mole of fatty acid oxidized. The fatty acid oxidase activity of the mitochondria prepared in isotonic sucrose was relatively stable to freezing and storage at low temperature.

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