On the Mechanism of $\beta$-Oxidation of Long Chain Fatty Acids by Liver Mitochondria from Normal and Alloxan-diabetic Rats*†

John A. Jones† and Melvin Blecher

From the Department of Biochemistry, Schools of Medicine and Dentistry, Georgetown University, Washington, D. C. 20007

(Received for publication, July 21, 1964)

An accepted mechanism for mitochondrial oxidation of fatty acids, i.e. $\beta$ oxidation, although inferred to apply also to long chain fatty acids ($>C_6$), has actually been elucidated only for relatively short chain acids ($<C_6$); only the activation (acyl coenzyme A synthetase) and initial dehydrogenation (acyl coenzyme A dehydrogenase) steps have been demonstrated for palmitate (1). The present paper reports the chemical synthesis of three $^{14}$C-labeled suspected intermediates in the $\beta$ oxidation of palmitate, and the oxidation of these derivatives to carbon dioxide and to acetoacetic acid by liver mitochondria obtained from normal and alloxan-diabetic rats. The comparison of normal with diabetic mitochondrial oxidation is of particular interest, since the mechanism (or mechanisms) behind the augmented oxidation of long chain fatty acids exhibited by intact diabetic rats, and by liver slices and perfused livers obtained from diabetic rats, remains obscure (2).

**EXPERIMENTAL PROCEDURE**

Methyl $\beta$-ketopalmitate-$1-{14}$C was prepared by condensation of myristoyl chloride with ethyl acetocetate-$1-{14}$C (Nuclear Research Chemicals, Orlando, Florida), followed by hydrolysis of the condensation product with methanolic hydrochloric acid. dl-$\beta$-Hydroxypalmitic acid-$1-{14}$C was synthesized by reaction of myristaldehyde with ethyl bromoacetate-$1-{14}$C (Volk Radiochemical Company, Chicago), followed by acid hydrolysis of the reaction product. trans-$\alpha$, $\beta$-Hexadecenoic acid-$1-{14}$C was isolated by vacuum distillation from the reaction mixture following pyrolysis of dl-$\beta$-hydroxypalmitic acid-$1-{14}$C in the presence of boric anhydride.1 Samples of the labeled $\beta$-hydroxy and $\alpha$, $\beta$-unsaturated derivatives, as well as palmitic acid-$1-{14}$C, were also obtained from Nuclear Research Chemicals. All compounds were characterized by elemental analysis, melting point behavior, optical rotation, and infrared spectroscopy. In addition, each compound yielded a single spot or peak, respectively, upon analysis of the methyl esters or free acids by thin layer chromatography on silica gel G and gas chromatography on diethylene glycol succinate; all of the applied radioactivity was confined to the single spot. Details of the synthesis and characterization of these compounds will be the subject of a separate communication.

Liver tissue, obtained from two 6- to 7-week-old male Holtzman rats, was homogenized in a solution composed of 0.01 M Tris buffer (pH 7.4)-0.001 M sodium EDTA-0.25 M sucrose, and was fractionated by standard methods (3, 4). Washed mitochondria were suspended in the buffer medium described above so that concentrations of mitochondrial protein (5) were approximately 10 mg per ml; 1 ml of this suspension was used per incubation mixture.

Diabetes was induced in rats by subcutaneous injection of alloxan monohydrate; a total of 180 mg per kg of body weight was given in two equal doses administered 24 hours apart to 48-hour fasted rats. Animals were used when they exhibited marked polyuria, polydypsia, and fasting blood glucose levels (6) exceeding 300 mg/100 ml; this occurred usually within 1 week after administration of alloxan. Normal and diabetic rats were fasted for 16 to 24 hours prior to killing.

The composition of the basic incubation mixture (Table I) is essentially that described by others (7), with the elimination of serum albumin. Each radioactive substrate, as a solution of its sodium salt in 0.012 M phosphate buffer, pH 7.4, was added to incubation vessels (25-ml flasks fitted with serum caps and hanging glass center wells) in a final concentration of 0.25 mM. Following incubation (see Table I for conditions), 0.3 ml of Hyamine hydroxide (Rohm and Haas; 1 M in methanol) was injected through the serum caps into the center well, and 0.3 ml of aniline hydrochloride-citric acid (0.5 g per ml each of aniline hydrochloride and citric acid in water) into the incubation mixture. The mixture was shaken for an additional 30 minutes to permit complete absorption of carbon dioxide by the Hyamine. Radioactive Hyamine carbonates were transferred to vials containing 10 ml of 0.4% 2,5-diphenyloxazole-0.05%, 1,4-bis(2-(5-phenyloxazolyl))benzene in toluene for determination of radioactivity in a Nuclear-Chicago liquid scintillation spectrometer; quench corrections were made by the channels ratio method. Fresh center well assemblies were then placed in incubation vessels, and 0.3 ml of Hyamine was injected into the center wells and 0.4 ml of aniline citrate (0.5 g per ml each of aniline hydrochloride and citric acid in water) into the incubation mixtures; the flasks were shaken for 60 minutes, during which period the carboxyl carbon of acetocetic acid, quantitatively removed as carbon dioxide, was trapped in the Hyamine; radioactivity was determined as above.

*Supported in part by Grant AM-06208 from the National Institutes of Health.

†Predoctoral Fellow, United States Public Health Service.

Data are taken from a dissertation to be submitted to the Graduate School of Arts and Sciences, Georgetown University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1 We are grateful to Dr. Carlo Colombini, University of Padova (Italy), for suggesting this reaction.
Table I

Oxidation of carboxyl-labeled palmitate and derivatives by mitochondria

In a final volume of 3.0 ml, incubation mixtures contained (in micromoles) succinate, 2.25; AMP, 6; KCl, 420; sucrose, 250; EDTA, 1; Tris buffer (pH 7.4), 10; phosphate buffer (pH 7.4), 6; and fatty acid substrate, 0.75 (20 000 c.p.m. per vessel for β-ketopalmitate-1-14C and 90 000 c.p.m. per vessel for the other three substrates); vessels contained 8 to 14 mg of mitochondrial protein. Incubation was conducted for 60 minutes at 37° with constant shaking and with air as gas phase. Values given are means ± standard errors of the means from 8 to 12 experiments with each substrate and with both types of mitochondria. Probability (p) values are given wherever results with diabetic mitochondria were significantly different from those with normal tissue.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Conversion to product</th>
<th>Normal CO₂ coated</th>
<th>Acetoacetate</th>
<th>Diabetic CO₂ coated</th>
<th>Acetoacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate-1-14C</td>
<td>3.74 ± 0.14</td>
<td>2.17 ± 0.18</td>
<td></td>
<td>4.51 ± 0.31 (p &lt; 0.01)</td>
<td>1.64 ± 0.12 (p &lt; 0.03)</td>
</tr>
<tr>
<td>Trans-α,β-Hexadecenoate-1-14C</td>
<td>1.67 ± 0.18</td>
<td>0.66 ± 0.17</td>
<td></td>
<td>1.49 ± 0.19</td>
<td>0.44 ± 0.04</td>
</tr>
<tr>
<td>Δ6,β-Hydroxypalmitate-1-14C</td>
<td>1.96 ± 0.27</td>
<td>0.14 ± 0.06</td>
<td>1.86 ± 0.17</td>
<td>0.21 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>β-Ketopalmitate-1-14C</td>
<td>49.0 ± 3.9</td>
<td></td>
<td>86.4 ± 4.3 (p &lt; 0.001)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are corrected for nonenzymatic release of carbon dioxide, which, with all substrates, amounted to less than 1% of that formed enzymatically.

|                        | Values given are calculated on the assumption that the carbonyl and carboxyl (isolated) carbons of acetoacetate contained equal amounts of radioactivity (8). Conversion of β-ketopalmitate-1-14C to acetoacetate was not determined, since aniline citrate also decarboxylated the former. |

RESULTS

As indicated by the data of Table I, all three suspected intermediates in the oxidation of palmitate, i.e. the trans-α,β-unsaturated, Δ6-β-hydroxy, and β-keto derivatives, were activated, oxidized to carbon dioxide, and converted to acetoacetic acid by liver mitochondria isolated from normal and alloxan-diabetic rats. Oxidation of the unsaturated and hydroxy derivatives to carbon dioxide occurred to about one-half and one-third of the extent of oxidation of palmitate in mitochondria of normal and diabetic rats, respectively; if, however, only the L(+)-isomer of Δ6,β-hydroxypalmitate was oxidized, as may be the case (1), then the oxidation of β-hydroxypalmitate by mitochondria of normal, but not diabetic, rats may have occurred to an extent similar to that of palmitate. In striking contrast to the above results, oxidation of β-ketopalmitate to carbon dioxide exceeded that of palmitate by over an order of magnitude in mitochondria of both normal and diabetic rats.

With mitochondria of both normal and diabetic rats, the conversion of the carboxyl carbon atoms of trans-α,β-hexadecenoate and β-hydroxypalmitate to acetoacetate occurred to a much smaller extent than conversion of the carboxyl carbon of palmitate. As noted in the legend to Table I, conversion of β-ketopalmitate to acetoacetate could not be determined by the procedures employed.

The data of Table I reveal a marked increase (1.8-fold) in the oxidation of β-ketopalmitate to carbon dioxide by mitochondria of diabetic, as compared to normal, rats; in addition, there was a less marked, but statistically significant, increase (1.2-fold) in the oxidation of palmitate to carbon dioxide in diabetic tissue. In contrast, oxidation of trans-α,β-hexadecenoate and β-hydroxypalmitate occurred to similar extents in both types of mitochondria.

The incorporation of isotope from trans-α,β-hexadecenoate and from β-hydroxypalmitate into acetoacetate was essentially the same in experiments with mitochondria of both normal and diabetic rats; however, there was a statistically significant decrease in the conversion of the carboxyl carbon of palmitate into acetoacetate in mitochondria of diabetic rats. Possible differences between mitochondria of normal and diabetic rats in the conversion of β-ketopalmitate to acetoacetate could not be explored by the techniques employed.

DISCUSSION

This paper reports the chemical synthesis of three 14C-labeled suspected intermediates in the mitochondrial β oxidation of a long chain fatty acid, palmitic acid. The observation that each of the derivatives, as its sodium salt, was oxidized to carbon dioxide and converted to acetoacetic acid by rat liver mitochondria indicates that the activation enzyme (or enzymes) for all three compounds is (are) present in liver mitochondria, and suggests that each compound is, or is convertible to, a true intermediate in the β oxidation of palmitate. In this connection, it is of interest that, while this report was in preparation, Davisoff and Korn (9) reported the formation of trans-α,β-hexadecenoate, n(-),β-hydroxypalmitate, and both cis- and trans-β,γ-hexa-deenoate from palmitoyl-CoA by enzyme preparations from Dictyostelium discoideum and by guinea pig liver mitochondria.

The differences in rates of oxidation between palmitate, trans-α,β-hexadecenoate, Δ6-β-hydroxypalmitate, and β-ketopalmitate observed in the present studies may reflect one or more of the following factors: (a) rates of penetration of mitochondria by the substrates; (b) rates of activation, perhaps to thioesters; (c) rates of activity of the specific enzyme oxidizing (or cleaving) each intermediate; and (d) rates of reduction of these derivatives to saturated fatty acids. As an example of Factor c above, the strikingly great oxidation of β-ketopalmitate to carbon dioxide observed in the present studies may be a reflection of the equilibrium constant for the β-ketoacyl-CoA thiolase reaction, which, at least in the case of short chain β keto fatty acids, is far in the direction of cleavage (K_eq with acetoacetyl-S-CoA is 6 × 10^6) (10). Studies are in progress with preparations of solubilized liver mitochondria in an effort to explore the above mentioned factors.

Of particular interest is the striking increase in the oxidation
of \( \beta \)-ketopalmitate, and the less marked increase in palmitate oxidation, observed in the present studies with diabetic liver mitochondria. The present observations may contribute to an explanation of the augmented oxidation of fatty acids observed in intact diabetic rats and in liver slices and perfused livers obtained from diabetic rats (2). In contrast to the present results, it has been reported previously (11) that, in alloxan diabetes, activities of acyl dehydrogenase, \( \beta \)-hydroxyacyl dehydrogenase, and \( \beta \)-ketoacyl thiolase were increased by 115, 39, and 17\%, respectively; however, it should be emphasized that these experiments were performed with mitochondrion-free liver homogenates and the substrates employed were thioesters of short chain (\( C_4 \)) fatty acids, and thus the present and previous experiments may not be comparable.

SUMMARY

Three suspected intermediates in the \( \beta \) oxidation of palmitate, trans-\( \alpha \),\( \beta \)-hexadecenoate, dl-\( \beta \)-hydroxypalmitate, and \( \beta \)-ketopalmitate, were chemically synthesized labeled with \( ^{14} \text{C} \) in their carboxyl groups, and were shown to be oxidized to carbon dioxide and to acetoacetic acid by liver mitochondria obtained from normal and diabetic rats. Oxidation of \( \beta \)-ketopalmitate-\( L^{14} \text{C} \) to carbon dioxide, which occurred in normal mitochondria to an extent which was 12- to 20 fold greater than that of the other three substrates, was increased almost 100\% in liver mitochondria obtained from diabetic animals; the oxidation of palmitate-\( L^{14} \text{C} \) was also increased above normal in diabetic liver mitochondria.

Acknowledgment—We acknowledge the technical assistance of Mr. Greene Paschal.

REFERENCES

On the Mechanism of β-Oxidation of Long Chain Fatty Acids by Liver Mitochondria from Normal and Alloxan-diabetic Rats

John A. Jones and Melvin Blecher