The Metabolism of Cyclopropane Fatty Acids by *Tetrahymena pyriformis*  

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SUMMARY

Cyclopropane fatty acids are readily metabolized by whole cells of *Tetrahymena pyriformis* when the fatty acids are presented either as components of intact *Escherichia coli* cells or as free fatty acids added to the medium. cis-11,12-[methylene-14C]Methylenoctadecanoic acid was degraded with the production of 14CO2 by whole *T. pyriformis* cells or a particulate fraction derived from them. In the presence of malonate, [2-14C]Acetate was formed. A pathway for degradation of the cyclopropane fatty acids, requiring only minor modification of the usual β oxidation pathway, is proposed.

**EXPERIMENTAL PROCEDURE**

**Materials**

Zinc-copper couple, methylene iodide, and nicotinamide were products of Eastman Kodak Co., Rochester, N. Y. Fatty acid methyl esters having even carbon numbers of 8 to 20, the methyl esters of cis-9,10-methylenoctadecanoic, vaccenic, and oleic acids and methyl [U-14C]oleate (750 mCi per mmole) were purchased from Applied Science Laboratories, State College, Pa. [1,3-14C]Acetone (26.8 mCi per mmole) was obtained from Amersham-Searle, Arlington Heights, Ill., and [U-14C]palmitic acid (0.05 mCi/16 μg) was obtained from New England Nuclear Corp., Boston, Mass. 1,4-Bis(4-(4-phenyl-5-pyrazolyl)azo)benzene (dimethyl POPOP), 2,5-diphenyloxazole (PPO), and 2,5-bis(5-tert-butylbenzoazolyl)thiophene (BBOT) were purchased from Packard Instrument Co., Inc., Downers Grove, Ill., and Cab-O-Sil was purchased from Research Products International Corp., Elk Grove Village, Ill. ATP, NAD, aminid, and di-thiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo., CoA and L-carnitine hydrochloride from Calbiochem, Los Angeles, Cal., and bovine serum albumin fraction V from Pentex Inc., Kankakee, Ill. All solvents were reagent grade and were redistilled.

**Preparation of [14C]Cyclopropane Fatty Acids**—Standard synthetic procedures were used, with certain modifications made necessary by the small quantities involved. A detailed description of these modifications is available (5), and a brief description of the procedure is provided here. [1,3-14C]Acetone (250 mCi) was diluted with unlabeled acetone and 14CH3 was made by reaction with I2 under alkaline conditions. The 14CH3 was reduced to 14CH4 with Na2AsO3 (6) and the 14CH4 was added to the double bond of methyl vaccenate using the Simmons-Smith reaction (7). The product, methyl cis-11,12-[methylene-14C]methyleneoctadecanoate, was purified by argentation thin layer chromatography (8), followed by preparative gas-liquid chromatography (9). The product had the same gas chromatographic retention time as methyl vaccenate purchased from Applied Science Laboratories. Methyl cis-9,10-methylenoctadecanoate was prepared from methyl [U-14C]oleate and unlabeled CH3I by the same method; the product has a mass spectrum identical to that reported for the same compound by Wood and Reiser (2). Before use, the methyl esters were saponified by refluxing for 2 hours in 4.0 ml of 5% KOH in 50% methanol, followed by acidification with 1.5 ml of 6.0 N HCl and extraction with ethyl ether. After removal of the solvent, the fatty acids were redissolved in 95% ethanol.

**Culture of Microorganisms**—*T. pyriformis* (GL) was obtained from Dr. D. Outka, Iowa State University. The organisms were ordinarily grown on a medium containing 2% proteose peptone and 0.1% yeast extract at 25° with shaking. *Escherichia coli* (BFG) was obtained from Dr. J. Horowitz, Iowa State University. Cells with a high cyclopropane fatty acid content were obtained by growth on a glucose and salts medium (10) for 24
Hours at 37° in a gyratory shaker. For experiments in which a T. pyriformis culture was to be added to the bacterial culture, the E. coli medium was supplemented with the following salts (concentrations given in milligrams per 100 ml of medium): CaCl2·2H2O, 5; Fe(NH4)2(SO4)·6H2O, 2.5; FeCl3·6H2O, 0.125; MnCl2·4H2O, 0.05; ZnCl2, 0.005; CuCl2·2H2O, 0.5.

Degradation of cis-11,12-[Methylene-14C]Methylenoctadecanoic Acid in Vivo—An apparatus was prepared in which T. pyriformis could be grown with a stream of CO2-free air sweeping respired CO2 into a flask containing 25% KOH, in which the CO2 was trapped. To 100 ml of culture medium in this apparatus were added 2.4 × 10^-6 pmole of cis-11,12-[Methylene-14C]methylenoctadecanoic acid (28 μCi per mmole) in 20 μl of 95% ethanol. After the E. coli medium was supplemented with the following salts as described by Rendina (13), and the radioactivity in this fraction was determined by scintillation counting with Cab-O-Sil solution used to trap CO2 were removed at intervals, and the radioactivity was measured in 10 ml of Bray's liquid scintillation solution.

Position of Labeled Carbon in [14C]acetate—Four experiments were performed in which cis-11,12-[Methylene-14C]methyleneoctadecanoic acid was incubated with the 10,000 × g pellet obtained from T. pyriformis as described in the caption for Table II. Four 3-ml reaction mixtures in 25-ml Erlenmeyer flasks were used in each experiment. The incubation medium was supplemented with 5.0 mm malonate. The radioactive acetate was isolated as described earlier except that 10 μmole of unlabeled acetate were added to each flask as carrier. The column fractions containing acetate from each experiment were pooled and dried under vacuum after the addition of 2.0 ml of 0.2 n ethanolic KOH. The samples collected from all experiments were pooled and dissolved in water to a total volume of 5.0 ml. An aliquot of 0.25 ml was transferred to a liquid scintillation vial and 0.25 ml of 2.5 n KOH was added to it. Fifteen milliliters of Cab-O-Sil gel then were added and radioactivity was measured.

The rest of the sample was transferred to a 25-ml reaction flask and dried under vacuum on a rotary evaporator. Five milligrams of unlabeled sodium acetate were added, and the sample was degraded by the Schmidt reaction (19).

RESULTS AND DISCUSSION

E. coli cells harvested in the stationary phase contain substantial amounts of cis-9,10-methylenedecanoic acid and cis-11,12-methylenoctadecanoic acid (1). T. pyriformis fatty acids do not include any components with gas chromatographic retention times close to those of the cyclopropane fatty acids so the disappearance of the cyclopropane fatty acids as the protozoa feed upon and digest the bacterial cells is easily seen (Fig. 1). In the first fatty acid profile of Fig. 1, the pattern is essentially that of E. coli alone. After three days a large peak between C17 and C18 cyclopropane fatty acids and C15, due to polyunsaturated C15 acids, appears while the C17 and C18 cyclopropane fatty acids decline in amount. After 5 days the cyclopropane fatty acids have nearly disappeared. There is no indication of the accumulation of C11 and C13 cyclopropane fatty acids, as occurred when cyclopropane fatty acids were fed to rats (2). These samples represent equal volumes of well stirred culture and the results cannot be explained by settling out of the bacterial cells.

Confirmation that metabolism of the cyclopropane fatty acids by T. pyriformis included degradation of the cyclopropane ring was obtained by growing the organism in proteose-peptone medium to which a cyclopropane fatty acid, labeled with 14C in the ring methylene carbon, was added. 14CO2 was evolved throughout the 7 days of the experiment (Table I). At the end of 7 days, total lipids were extracted, and glycogen was isolated from the lipid-free residue. The fraction of the total 14C added that was recovered in each of the materials isolated was: CO2, 9.1%; lipid,
FIG. 1. Gas chromatographic elution profiles for the methyl esters of fatty acids from a culture of Tetrahymena pyriformis feeding upon Escherichia coli cells. CYC, cyclopropane fatty acids.

TABLE I
Evolution of \(^{14} \text{CO}_2\) during metabolism of cis-11,12-[methylene-\(^{14} \text{C}\)]-methyleneoctadecanoic acid by Tetrahymena pyriformis cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Total (^{14} \text{CO}_2) evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1010</td>
</tr>
<tr>
<td>2</td>
<td>1505</td>
</tr>
<tr>
<td>3</td>
<td>2037</td>
</tr>
<tr>
<td>4</td>
<td>4199</td>
</tr>
<tr>
<td>5</td>
<td>5813</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
</tr>
<tr>
<td>7</td>
<td>8113</td>
</tr>
</tbody>
</table>

33.6%; and glycogen, 1.3%. The incorporation of label into glycogen could come from the conversion of \([^{14} \text{C}]\)acetate to glucose (20) or from fixation of \(^{14} \text{CO}_2\).

A particulate fraction which includes the mitochondria from cell homogenates (14) was prepared and found to oxidize palmitic acid readily (Table II). Addition of malonate reduced the \(^{14} \text{CO}_2\) production drastically and allowed the recovery of a small amount of \([^{14} \text{C}]\)acetate. When a similar experiment was performed with methylene-labeled cyclopropane fatty acid as the substrate, \(^{14} \text{CO}_2\) was also produced, and \([^{14} \text{C}]\)acetate was isolated when its oxidation was inhibited by malonate (Table III). The yield of \(^{14} \text{CO}_2\) from palmitic acid was 12.5% of the theoretical in the absence of malonate, while from the cyclopropane fatty acid it was 1.8%. The low yield in the latter case may be caused by relatively slow metabolism of the cyclopropane ring, or it may merely reflect the fact that release of labeled product from this substrate requires six turns of the \(\beta\) oxidation cycle while a single turn releases \([^{14} \text{C}]\)acetate from \([U-^{14} \text{C}]\)palmitate.

A cyclopropane fatty acid labeled at all carbons except the ring methylene carbon was prepared from methyl \([U-^{14} \text{C}]\)oleate and unlabeled methylene iodide to determine whether the methyl-terminal end of the cyclopropane fatty acid is converted to propionate. Even in the presence of avidin, only a very small amount of labeled propionate was found (Table III), although a substantial amount of \([^{14} \text{C}]\)acetate was isolated when malonate was present. Acetate accumulated from four separate reactions of cis-11,12-[methylene-\(^{14} \text{C}\)]methyleneoctadecanoic acid with 10,000 X g particle preparations was degraded by the Schmidt reaction. Starting with a sample containing 1662 dpm of \(^{14} \text{C}\), 3 dpm were recovered from the carboxyl group and 834 dpm from the methyl group. We conclude that the ring methylene carbon of the cyclopropane fatty acid substrate appears exclusively in the methyl group of acetate.

Schiller and Chung (21, 22) studied the metabolism of cyclopropanecarboxylic acid by Fusarium oxysporum and concluded the cyclopropane ring was opened by a reaction or reaction sequence in which the elements of water are added across the ring. The validity of this reaction as a model for the metabolism of the cyclopropane ring in the cyclopropane fatty acids is doubtful since the intermediate products of \(\beta\) oxidation of the cyclopropane fatty acids have at least one methylene group between the ring

<table>
<thead>
<tr>
<th>Supplement to reaction medium</th>
<th>(^{14} \text{CO}_2) (cpm)</th>
<th>([^{14} \text{C}])Acetate (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2008</td>
<td>2</td>
</tr>
<tr>
<td>5 mM malonate</td>
<td>N.D.*</td>
<td>2</td>
</tr>
<tr>
<td>10 mM malonate</td>
<td>N.D.*</td>
<td>0</td>
</tr>
</tbody>
</table>

Expt. 2

<table>
<thead>
<tr>
<th>Supplement to reaction medium</th>
<th>(^{14} \text{CO}_2) (cpm)</th>
<th>([^{14} \text{C}])propionate (cpm)</th>
<th>([^{14} \text{C}])Acetate (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10,098</td>
<td>130</td>
<td>600</td>
</tr>
<tr>
<td>5 mM malonate</td>
<td>10,338</td>
<td>130</td>
<td>600</td>
</tr>
<tr>
<td>100 (\mu)g of avidin</td>
<td>9,943</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>200 (\mu)g of avidin</td>
<td>9,434</td>
<td>15</td>
<td>696</td>
</tr>
<tr>
<td>100 (\mu)g of avidin, 5 mM</td>
<td>N.D.</td>
<td>12</td>
<td>31</td>
</tr>
</tbody>
</table>

* N.D., not determined.
and the carbonyl group. The reactivity of such compounds with respect to Michael-type addition of water, or the other reactions suggested by Schiller and Chung (21, 22) to account for the opening of the cyclopropane ring, would be expected to be much less than that of compounds such as cyclopropanecarboxylic acid, in which the ring is adjacent to the carbonyl group. We can account for our results by a simple modification of β oxidation as shown in the following reaction sequence:

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_2\text{CH} & \rightarrow \text{CH} = \text{CH} - \text{CO} - \text{CoA} \\
\text{CH}_2(\text{CH}_2)_n\text{CH} & \rightarrow \text{CH} = \text{CH} - \text{CO} - \text{CoA} \quad + 4 \text{CH}_2\text{CO} - \text{CoA} \\
\text{CH}_2(\text{CH}_2)_n\text{CH} & \rightarrow \text{CH} - \text{CH} - \text{CO} - \text{CoA} \\
\text{CH}_2(\text{CH}_2)_n\text{CH} & \rightarrow \text{CH} - \text{CO} - \text{CoA} \quad + \text{CH}_2\text{CO} - \text{CoA} \\
\text{CH}_2\text{CH}_2\text{CO} & \rightarrow \text{CH}_2\text{CO} - \text{CoA} + 2\text{CH}_2\text{CO} - \text{CoA} + \text{CH}_3\text{CO} - \text{CoA}.
\end{align*}
\]

Step 4 may be nonenzymatic since the tautomerization of cyclopropanols to open chain carbonyl compounds occurs under mild conditions (23).

REFERENCES

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