ω-Cyclohexyl Fatty Acids in Acidophilic Thermophilic Bacteria

STUDIES ON THEIR PRESENCE, STRUCTURE, AND BIOSYNTHESIS USING PRECURSORS LABELED WITH STABLE ISOTOPES AND RADIOISOTOPES*

(Received for publication, March 17, 1975)

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ω-Cyclohexyl undecanoic acid and ω-cyclohexyl tridecanoic acid were found in 10 strains of acido-thermophilic bacteria isolated from different Japanese hot springs. These unusual fatty acids were found in the esterified form in glyceride type complex lipids and constituted 74 to 93% of the total fatty acids in the bacteria.

The fatty acids other than ω-cyclohexyl fatty acids found were 14-methyl hexadecanoic acid (3 to 15%) and 15-methyl hexadecanoic acid (1 to 6%), and trace amounts of straight chain and methyl-branched tetra- and penta-decanoic acids.

 Biosynthesis of ω-cyclohexyl fatty acids increased with increase in the concentration of glucose in the culture medium.

The metabolism of ω-cyclohexyl fatty acids was studied using deuterium-labeled precursors by mass fragmentation analysis. The deuterium of [2-D]glucose was specifically incorporated into position 2 of the cyclohexyl ring of the fatty acids, indicating that the ring was synthesized from the glucose molecule.

Radioactivity was efficiently incorporated into the ω-cyclohexyl fatty acids from labeled glucose, shikimate, and cyclohexyl carboxylate. These findings indicate that ω-cyclohexyl fatty acids are synthesized with glucose through shikimic acid and probably cyclohexyl carboxyl-CoA derivative as the intermediates.

Acidophilic-thermophilic bacteria are characterized by their tolerance to high temperature and acidity (45-70°, pH 2 to 5). Uchino and Doi (1) first reported the isolation of several strains of aerobic spore-forming acido-thermophiles from hot springs in the northern part of Japan. Morphologically similar organisms were isolated from the geyser basin of Yellowstone National Park, and from soil of the Volcano National Park, Hawaii (2) and of the hot springs in Pisciarelli, Italy (3). It was proposed that these organisms should be classified in a new species named Bacillus acidocardarius.

In such acid- and heat-resistant organisms, the membranes may have special characters for maintaining the organizational integrity of the cell contents. This seems very probable, since the pH optima of intracellular enzymes are not as low as the optimum pH for cell growth, so the membranes may play an important role as a barrier against the acidic environment.

There are several reports of the presence of iso- and anti-iso-branched fatty acids, especially 15-methyl hexadecanoic acid as components of thermophiles (4-8). Moreover, De Rosa et al. (3) reported the presence of fatty acids containing the cyclohexane ring from acido-thermophiles isolated from Italian hot springs.

We investigated the fatty acids of 10 strains of acido-thermophilic bacteria isolated from different hot springs and geyser basins in Japan. These acido-thermophiles may be strains of Bacillus acidocardarius, but this was only deduced from morphological examination.

This paper described studies on the structures of these unusual fatty acids and their metabolism examined using precursors labeled with stable isotope (D) and radioisotope (14C).

EXPERIMENTAL PROCEDURE

Materials

The liquid and solid phases used for gas-liquid chromatography were purchased from Gascho-Kogyo (Tokyo). Stearic acid (purity 99%), used as an internal standard, was obtained from Sigma Chemical Co. (St. Louis, Mo.). Sodium [1-14C]acetate (0.05 mCi/mmol), [U-14C]glucose (0.05 mCi/mol), [U-14C]shikimic acid (10 μCi/mol), and [1-14C]cyclohexyl carboxylic acid (10 μCi/mmol) were purchased from Daiichi Chemical Co. (Tokyo). Shikimic acid used for synthesis of [2-14C]shikimic acid, was obtained from Wako Chemical Co. (Tokyo), and deuterium-labeled sodium hydroxide, deuterium oxide, [1-D]-acetate acid, sodium borodeuteride, and [1-D]chloroform were products of Merck Darmstadt (Germany).

Methods

All strains of acidophilic-thermophilic bacteria were cultured at 55-60° with shaking in medium containing KH2PO4 (0.01%),
the method of Bligh and Dyer (11). The total lipid extracted was subjected to methanolysis, and the fatty acid methyl esters were

Culture medium (10 to 50 pCi/50 ml). Lipid was extracted from cells by centrifugation. The cells were washed thoroughly and lyophilized. In some experiments, deuterium-labeled precursors were added to the culture medium. The fatty acid methyl esters were analyzed by radio-gas chromatography.

RESULTS AND DISCUSSION

Presence and Structure of ω-Cyclohexyl Fatty Acids

All 10 strains of acido-thermophilic bacteria isolated from different Japanese hot springs and geyser basins contained 74 to 93% (of the total fatty acid content) of ω-cyclohexyl fatty acids (Table I).

Results on the gas chromatographic separation of these fatty acids are shown in Fig. 1. The purities of the 11-cyclohexyl undecanoate and 13-cyclohexyl tridecanoate separated by preparative gas chromatography were 80.7% and 86.5%, respectively.

The infra red spectrum of the ω-cyclohexyl fatty acids (Fig. 2) shows the C=O-scissoring vibration of the cyclohexane ring (1455 cm⁻¹). The absorption bands at 892 and 840 cm⁻¹ also provide strong evidence for the presence of a cycloalkane ring.

The proton nuclear magnetic resonance spectra (Fig. 3) of the ω-cyclohexyl fatty acids shows the methylene protons of a cycloalkane ring (broad signal around 1.64 ppm) and indicates the absence of terminal methyl protons. The small signal at 0.5 ppm, therefore, is designated as methyl protons, since the methyl protons of a terminal methanol group would give rise to another signal at 0.8 ppm.

<table>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td>Fatty acid compositions of 10 acidophilic-thermophilic bacterial strains</td>
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<tr>
<td>Strain</td>
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<tr>
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<tr>
<td>Fatty acid content</td>
</tr>
<tr>
<td>Total fatty acid</td>
</tr>
</tbody>
</table>

*The abbreviations used are: 13:n,n-tridecanoic acid; 15:i,13-pentadecanoic acid; 15:a,12-pentadecanoic acid; 16:i,14-pentadecanoic acid; 16:n,hexadecanoic acid; 17:i,15-hexadecanoic acid; 17:a,14-hexadecanoic acid; 11:cy, ω-cyclohexyl undecanoic acid; 13:cy, ω-cyclohexyl tridecanoic acid; ω-cyclohexyl fatty acids.

†Percentages were calculated as % areas by gas-chromatographic analysis.

†T, less than 0.4% of the total fatty acids.

FIG. 1. Gas-liquid chromatography of ω-cyclohexyl fatty acids. Lyophilized cells were subjected to methanolysis in 3% HCl in dry methanol at 100°C for 4 hours. The fatty acid methyl esters were extracted with light petroleum ether and analyzed in a 25% polyethylene glycol succinate column (3.5 mm) at 170°C. Peak A, 11-cyclohexyl undecanoate; B, 13-cyclohexyl tridecanoate.
FIG. 2. Infrared spectrum of methyl 13-cyclohexyl tridecanoate recorded in a Hitachi 215 spectrometer.

FIG. 3. Proton nuclear magnetic resonance spectrum of isolated methyl 11-cyclohexyl undecanoate recorded in chloroform-D at room temperature. Fatty acid methyl esters were recorded by preparative gas-liquid chromatography (column 4% SE-30, 1 m).

FIG. 4. GC-MS spectrum of methyl 11-cyclohexyl undecanoate.

0.95 ppm could be due to the terminal methyl protons of a trace of 14-methyl hexadecanoic acid contaminating the 11-cyclohexyl undecanoate (about 15%).

The fragment ions at m/e 83, 69, 55, and 41 shown in the GC-MS spectra (Figs. 4 and 6) of the ω-cyclohexyl fatty acid methyl esters give evidence of fragmentation of the cyclohexane ring. The intensity of the fragment ions at m/e 41, 55, 69, and 83 was the same as in the mass spectrum of authentic cyclohexyl carboxylate, which also indicated the cyclohexane ring fragmentation. The molecular weights of these two unidentified ω-cyclohexyl fatty acid methyl esters were determined as 282 and 310, respectively. The fragmentation diagrams of the fatty acid methyl esters of the compounds with molecular weights of 282 and 310 are shown in Fig. 5 (top) and Fig. 6, respectively.

These findings all indicate that the unusual fatty acids are 11-cyclohexyl undecanoic acid and 13-cyclohexyl tridecanoic acid.

Schoct and Begemann identified 11-cyclohexyl undecanoic acid as a very minor component of butterfat (12). Hansen (13) suggested that this compound might be of bacterial origin, since it constituted about 3% of the total fatty acids in rumen bacteria. ω-Cyclohexyl undecanoic acid and tridecanoic acid have also been found in Bacillus acidocardarius (3, 14), and the 10 strains we isolated from Japanese hot springs may be taxonomically similar organisms.

Effects of Culture Conditions on Biosynthesis of ω-Cyclohexyl Fatty Acids

The effects of temperature and pH on the biosynthesis of ω-cyclohexyl fatty acids are diagrammatically shown in Fig. 7, indicating that synthesis was highest under the optimal conditions for growth of the bacteria (pH 3, 60°C).

De Rosa et al. (14) discussed the effect of culture conditions on the fatty acid composition of a strain of B. acidocardarius from Agnano (Italy). They considered that demand for energy...
Fig. 7. Effects of temperature and pH on the biosynthesis of ω-cyclohexyl fatty acids. Some of the data from 30 experiments under different culture conditions are shown. The amounts of fatty acids were determined as percentage areas by gas-liquid chromatography.

and the availability of oxygen have independent effects on the pattern of carbon utilization in this organism. They suggested that at low pH, ion-pumping mechanisms demand a considerable amount of energy (since the intracellular pH is almost neutral), and that under these conditions metabolism favors production of ω-cyclohexyl fatty acids. At higher pH values, this energy demand is less, and carbon metabolism favors oxidative synthesis of acyclic acid at low temperature and reductive synthesis of ω-cyclohexyl fatty acids at high temperature. The solubility of atmospheric oxygen in water at 70°C is only two-thirds of that at 50°C.

In 30 experiments, we could not show any clear effects of pH and temperature on the metabolism of ω-cyclohexyl fatty acids. However, results indicated that under conditions favoring spore formation (extremely high or low pH and temperature), synthesis of ω-cyclohexyl fatty acids was low.

Glucose had a marked effect on the biosynthesis of ω-cyclohexyl fatty acids. Synthesis increasing with the amount of glucose in the medium. Glucose did not affect the synthesis of acyclic acid (i.e. 14-methyl hexadecanoic acid) (Fig. 8). Thus the synthesis of ω-cyclohexyl fatty acid is probably related to glucose metabolism.

Table II

Incorporation of deuterium from D-labeled precursors

D-labeled precursors were added to the culture medium and the cells were harvested in the stationary growth phase. Values represent per cent intensity of the true incorporation of D into ω-cyclohexyl fatty acids.

<table>
<thead>
<tr>
<th>Parent ion</th>
<th>11-Cyclic</th>
<th>13-Cyclic</th>
</tr>
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<tbody>
<tr>
<td>G-2-D</td>
<td>Me-G-6-D</td>
<td>2-D-S</td>
</tr>
<tr>
<td>(m/e)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>41</td>
<td>13.3</td>
<td>12.1</td>
</tr>
<tr>
<td>69</td>
<td>11.1</td>
<td>2.1</td>
</tr>
<tr>
<td>87</td>
<td>5.4</td>
<td>T</td>
</tr>
<tr>
<td>199</td>
<td>11.8</td>
<td>7.2</td>
</tr>
<tr>
<td>(M + 2)</td>
<td>20.1</td>
<td>T</td>
</tr>
</tbody>
</table>

The abbreviations used are: G-2-D, D-[2-D]glucose; Me-G-6-D, methyl-[6-D],glucoside; 2-D-S, [2-D]-shikimic acid; T, trace, less than 2%.

Biosynthesis of ω-Cyclohexyl Fatty Acids

Studies with Stable Isotopic Precursors—Fig. 9 shows the mass spectra of D-labeled methyl 11-cyclohexyl undecanoate (top) and methyl 13-cyclohexyl tridecanoate (bottom). The strong signals of fragment ions at m/e 41 + 1, 55 + 1, 69 + 1, and 83 + 1 indicate that the deuterium was incorporated into the cyclohexane ring from the deuterium at C-2 of the glucose molecule. Results also suggested that the C-2 position of the alkyl cyclohexane ring was specifically labeled with [2-
TABLE III
Incorporation of radioactivity into acyclic and cyclic fatty acids

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific activity (dpm/mol) x 10^-6</th>
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<tbody>
<tr>
<td></td>
<td>15:ante-iso</td>
</tr>
<tr>
<td>[1-14C]Acetate</td>
<td>1880</td>
</tr>
<tr>
<td>[U-14C]Glucose</td>
<td>773</td>
</tr>
<tr>
<td>[U-14C]Shikimic acid</td>
<td></td>
</tr>
<tr>
<td>[1-14C]Cyclohexyl carboxylic acid</td>
<td></td>
</tr>
</tbody>
</table>

*Dpm per mol of fatty acids determined with stearic acid as an internal standard.

Percentages of deuterium incorporated into 11-cyclohexyl undecanoic acid and 13-cyclohexyl tridecanoic acid from [2-D]glucose, calculated from the intensity of the ions higher than molecular ion (M + 1, M + 2, M + 3, ... ) divided by the intensity of the molecular ion are 78.4% and 90.5%, respectively. Those from methyl-[6-D]glucoside are 27.2% and 12.9% and from [2-D]shikimate are 3.2% and 4.9%, respectively. The lower incorporation of the deuterium from shikimate may be due to the incorporation only to the cyclohexane ring to the n-alkyl chain.

Studies with Radioisotopic Precursors—Table III shows the incorporation of 14C from the labeled precursors. Incorporation of radioactivity into ω-cyclohexyl fatty acids was high using [U-14C]shikimic acid and [1-14C]cyclohexyl carboxylic acid. Those results suggest that ω-cyclohexyl fatty acids were synthesized by the reduction of the shikimate and elongation of the alkyl chain of cyclohexyl carboxylate. Results suggested that simple chain elongation on the cyclohexyl carboxylate occurred rather than that the starter unit of cyclohexyl propionate was formed by decarboxylation of the prephenate, the intermediate of phenylalanine biosynthesis. De Rosa et al. also reported the 3H incorporation from the shikimate into the cyclohexyl fatty acids found in the Italian strain of B. acidocardarius (15).

Thus studies with both deuterium-labeled and 14C-labeled precursors showed that ω-cyclohexyl fatty acids are synthesized from glucose through shikimate and cyclohexyl carboxylate, as shown in Fig. 10.

Many bacterial fatty acids (i.e., iso- or ante-iso-branched cyclopropane fatty acids and 10-methyl-branched acids) are known to be derived from the corresponding amino acids, such as valine, isoleucine, leucine, or methionine, and thus the fatty acid compositions of the bacteria are linked to their amino acid

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**Fig. 10.** Scheme of biosynthesis of ω-cyclohexyl fatty acids. Circled protons indicate positions labeled by the deuterium of [2-D]glucose or [2-D]shikimic acid.
metabolism (16). However, the metabolism of \( \omega \)-cyclohexyl fatty acids are related to the metabolism of glucose, not amino acids. This may represent an adaptation of acidothermophilic bacteria to live in hot springs with low contents of amino acid nutrients.

The \( n \)-alkyl cyclohexanes found in a number of geological deposits have hitherto been thought to result from chemical ring formation of unsaturated fatty acids of biological origin at high temperature and pressure, since their biochemical precursors were unknown (17, 18). However, the presence of large amounts of \( \omega \)-cyclohexyl fatty acids in Japanese strains of acido-thermophiles as well as in \( B. \ acidocardarius \) isolated from Italian and American hot springs indicates that \( n \)-alkyl cyclohexane were probably derived from \( \omega \)-cyclohexyl fatty acids of acido-thermophiles, if these bacteria existed in prehistoric times.

Acknowledgments—The authors are indebted to Dr. T. Oshima of Mitsubishi Kasei Institute of Life Sciences for providing the acidophilic thermophilic bacterial strains AT-6, BA-152, FA-1, BA-18, BA-151, BA-11, and BA-5; and to Dr. F. Uchino of Nagoya University for strains T-7, T-17, and TM-24. They are also grateful to Dr. S. Ando of the Tokyo Metropolitan Institute of Gerontology for recording NMR spectra. The authors also thank Mr. A. Miyagawa for his excellent technical assistance in part of this investigation.

REFERENCES

12. Schogt, J. C. M., and Begemann, P. H. (1965) J. Lipid Res. 6, 466–470
16. Lennarts, W. J. Advan. Lipid Res. 4, 181
Omega-cyclohexyl fatty acids in acidophilic thermophilic bacteria. Studies on their presence, structure, and biosynthesis using precursors labeled with stable isotopes and radioisotopes.

M Oshima and T Ariga