Unsaturated Fatty Acids in Microorganisms*

GÜNTER SCHEUERBRANDT AND KONRAD BLOCH

From the James Bryant Conant Laboratory, Harvard University, Cambridge, Massachusetts

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Reports from this laboratory have described the existence of two different pathways for the biosynthesis of long chain monounsaturated fatty acids by microorganisms. In yeast, oleic and palmitoleic acids are formed oxidatively from the corresponding saturated acids by processes requiring molecular oxygen and TPNH (1). The same conversions have been demonstrated in Mycobacterium phlei (2), in the blue-green alga Anabaena variabilis, and in Penicillium chrysogenum. The second mechanism for the synthesis of monounsaturated fatty acids, discovered in Clostridium butyricum (3, 4), is anaerobic and produces the olefinic double bonds in the course of the chain-lengthening process rather than by desaturation of the corresponding saturated acids. This nonoxidative pathway appears to be employed not only by the obligate anaerobes but also by various aerobic representatives of the order Eubacteiriales. In fungi, plants, and animal tissues, the predominant monounsaturated fatty acids are Δ⁹-hexadecenoic and Δ⁷-octadecenoic acids (5). On the other hand, as first shown by the work of Hofmann, Lucas, and Sax (6), cis-Δ⁹-octadecenoic acid is the principal monounsaturated acid of lactobacilli species. It was later found also in Agrobacterium tumefaciens (7), in a species of Group C Streptococcus (8), and in Escherichia coli (9). The purpose of the present investigation was to ascertain whether Δ¹₁-octadecenoic acid is a common constituent of bacterial lipids and whether the double bond structure of unsaturated fatty acids can be related to the mechanism by which they are synthesized. The findings reported in this paper indicate that such a correlation exists. In the course of this work, two monounsaturated fatty acids, not previously described, have been isolated from bacterial sources. Polyunsaturated fatty acids could not be detected in any of the organisms examined.

EXPERIMENTAL PROCEDURE

All microorganisms were grown on chemically defined media as summarized in Table I. Fatty acids were isolated by the procedure described in another publication from this laboratory (2). The hydrolysates therefore included the fatty acids from both bound and free lipids. Unsaturated fatty acids and hydroxy acids were separated from the saturated acids by the mercuric acetate method (3). Methanol was used instead of ethanol to avoid transesterification of the methyl esters during elution of the mercury complexes. In some instances the hydroxy acids were separated from the unsaturated fraction by gas-liquid chromatography on silicic acid (11). The unsaturated methyl esters were eluted with anhydrous methylene chloride and the hydroxy compounds, which were not examined further, were washed from the column with methylene chloride containing 3% methanol. Gas-liquid chromatography of the fatty acid methyl esters on a polyethylene glycol succinate column and the procedure for trapping individual fatty acid fractions have been described before (2, 8).

Assignments of molecular size and degree of unsaturation were made from the retention times (18). Since double bond isomers of long chain monounsaturated fatty acids are not ordinarily separable by gas-liquid chromatography, the position of the double bond was determined by oxidative cleavage of the fatty acid followed by gas chromatographic identification of the resulting dicarboxylic acids. The following procedure is an adaptation to the microscale of the permanganate-periodate method described by von Rudloff (19) for the oxidative degradation of unsaturated acids to mono- and dicarboxylic acids.

The methyl esters of the total unsaturated fatty acids were separated by gas-liquid chromatography into the C₁₄, C₁₆, and C₁₈ fractions (retention times, 0.330, 0.605, and 1.200, respectively, relative to methyl stearate = 1.000) and the chromatographic runs were repeated until a sufficient amount (20 to 200 μg) of each fraction had been collected. The relative weights of recovered fatty acids were estimated from the area under the peak recorded during chromatography. The relation between peak area and weight of collected material was established with a sample of C₁₄-methyl oleate of known specific activity. In the procedure used, 60 to 80% of the fatty acid ester injected into the chromatography column was recovered on collection. The condensed material was rinsed with ether into a 1-ml, round bottom volumetric flask and a small aliquot was redensitographed to check the purity of the isolated material. The remaining solution was brought to dryness in a stream of nitrogen. For samples containing about 100 μg of material, the following oxidation mixture was added: 0.040 ml of t-butanol; 0.027 ml of oxidant solution (2.065 g = 97.5 mmoles of NaIO₄ + 0.158 g = 2.5 mmoles of KMnO₄ in 100 ml of water); 0.012 ml of a 0.02 M K₂CO₃ solution; and 0.06 ml of water. The stopped flask was attached to the stirring rod of a Vibro-Mischer (Ag. für Chemie, Apparatebau, Zurich, and agitation for 2 hours at room temperature. The mixture was decolorized with a few crystals of NaHSO₃, 0.025 ml of 1 N KOH was added, and the solution was evaporated to dryness with a warm water bath in a stream of nitrogen. Hydrochloric acid (0.050 ml of 1 N solution) was added and the solution was again brought to dryness. Trituration of the residue with three 0.5-ml portions of low boiling petroleum ether removed the monocarboxylic acids, which were analyzed independently in some instances by chromatography of

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† W. J. Lennarz and K. Bloch, unpublished experiments.

‡ W. J. Lennarz and B. Alter, unpublished experiments.
the methyl esters at a column temperature of 82°C. The free dicarboxylic acids were extracted from the remaining residue by benzene and analyzed by gas-liquid chromatography; the column temperature was 175°C. The identity of the dicarboxylic acids were esterified by diazomethane distilled from a stock solution, concentrated, a drop of methanol was added, and the fatty acids were esterified by diazomethane distilled from a stock solution. After 5 minutes, the solution was brought to dryness and the dimethyl esters were dissolved in an appropriate amount of benzene and analyzed by gas-liquid chromatography; the column packing was polyethylene glycol succinate on Chromosorb B, and the temperature was 175°C. The identity of the dicarboxylic esters was established by comparison of the retention times with those of the authentic compounds. The retention times for the dicarboxylic acid esters were based on a value of 1.00 for methyl stearate, C17, 0.389; C18, 0.540; C19, 0.740; C20, 1.000; C21, 1.370.

The procedure was checked by oxidation of a 1:1 mixture of methyl oleate and methyl Δ⁴-octadecenoic acid.³ Methyl azelate and methyl undecanoate in equal amounts were the only detectable products in the dicarboxylic acid fraction. The yield was 85%.

RESULTS AND DISCUSSION

The analytical data obtained for the monounsaturated fatty acids of various microorganisms are summarized in Table II. The microorganisms are arranged in two groups according to the mechanism employed for the synthesis of unsaturated fatty acids of various microorganisms are summarized in Table II.

The microorganisms listed in Table II are divided into two groups according to the mechanism employed for the synthesis of unsaturated fatty acids of various microorganisms. The upper group consists of representatives of fungi, algae, mycobacteria, and pseudomonads. The lower group consists of bacteria, yeasts, and viruses.

The yield of cells was increased 7-fold when 100 ml of amino acid mixture (12) per liter of medium was added, and when the glucose solution was sterilized separately (suggested by P. E. Baronowsky). The simultaneous occurrence of oleic and palmitoleic acids in M. phlei is in line with the results of Cason and Tavs (20), who have shown that in the related Mycobacterium tuberculosis, the monounsaturated fatty acids are exclusively Δ⁹ compounds. However, M. phlei has now been found to contain in addition a Δ¹⁰-hexadecenoic acid which is in fact the principal monounsaturated fatty acid in this species. Δ¹⁰-Hexadecenoic acid has not been isolated previously from natural sources. About 2 mg of the C₁₈ monounsaturated fraction were isolated from the lipids of M. phlei by repeated gas-liquid chromatography, for further characterization. The infrared spectrum of the mixture of the Δ⁹ and the Δ¹⁰ isomers lacked the C-H deformation band at 965 cm⁻¹, characteristic of 1,2-disubstituted trans double bonds. Therefore the new Δ¹⁰ isomer which is the major component in the mixture must have the cis configuration.

In metabolic experiments with growing cultures of M. phlei (Table III) it was found that the Δ¹⁰-hexadecenoic acid, like palmitoleic acid, is formed from palmitic acid. From C₁₄-palmitoleic acid, only very small amounts of C₁₄ were incorporated into the Δ¹⁰ compound, indicating that this acid is formed by direct dehydrogenation of palmitic acid and not by isomerization of palmitoleate.

A distinctly different and more complex fatty acid pattern is found in the second group of microorganisms listed in Table II, all members of the order Eubacteriales. The principal C₁₈ acid in every one of these organisms is the Δ₁₀ isomer of oleic acid. Whether these octadecenoic acids have the cis or trans configuration has not been determined. Oleic acid was detectable only in the lipids of C. butyricum. The simultaneous occurrence of...
Δ'-octadecenoic and oleic acids has already been observed for the lipids of a Group C Streptococcus species (8) and of E. coli (9). In the C16 fraction of the bacterial lipids a Δ'-hexadecenoic acid is the major component. In two species of clostridia the Δ².C16 acid is accompanied by Δ' or Δ² isomers. A Δ²-hexadecenoic acid has previously been identified in a Streptococcus species (8), and Δ²-hexadecenoic acid (hypogaeic acid) is stated to be present in plant oils (21-23). Finally, Rhodospseudomonas spheroides and two of the clostridia examined contain a Δ³-tetradecenoic acid, not previously encountered in any natural source.

These fatty acid patterns are noteworthy in two respects: (a) A monounsaturated fatty acid of a given chain length can occur in the same organism in pairs of two positional isomers, the location of the double bond differing by two carbon atoms. (b) The presence of oleic acid in an unsaturated fatty acid of the general structure CH3(CH2)7CH=CH(CH2)n- COOH (n = 3, 5, or 7) without reduction or dehydrogenation of the double bond. It has been observed by Hofmann et al. (6) that the cis isomers of the series Δ²-C16, Δ'-C16, and Δ²-C16 have biotin-sparing activity in lactobacilli, substituting for Δ²-octadecenoic acid in this respect (24). Hofmann et al. (6) have suggested that these short chain acids exert their biotin-sparing effect either by being converted to Δ²-octadecenoic acid or by substituting for it metabolically. A detailed mechanism for the synthesis of Δ²-octadecenoic acid and homologous acids by microorganisms has been discussed by Kaneshiro and Marr (9).

The approximate content of unsaturated acids, in per cent of the total long chain acids, is given in parentheses. The presence of oleic acid in an unsaturated fatty acid of the general structure CH3(CH2)7CH=CH(CH2)n-COOH (n = 3, 5, or 7) without reduction or dehydrogenation of the double bond. It has been observed by Hofmann et al. (6) that the cis isomers of the series Δ²-C16, Δ'-C16, and Δ²-C16 have biotin-sparing activity in lactobacilli, substituting for Δ²-octadecenoic acid in this respect (24). Hofmann et al. (6) have suggested that these short chain acids exert their biotin-sparing effect either by being converted to Δ²-octadecenoic acid or by substituting for it metabolically. A detailed mechanism for the synthesis of Δ²-octadecenoic acid and homologous acids by microorganisms has been discussed by Kaneshiro and Marr (9).

**TABLE II**

**Positions of double bonds in unsaturated fatty acids of microorganisms**

The numbers refer to the chain length of dicarboxylic acids obtained on oxidation, which establishes the position of the double bond. The approximate content of unsaturated acids, in per cent of the total long chain acids, is given in parentheses.

<table>
<thead>
<tr>
<th>Organisms with aerobic mechanism</th>
<th>C16</th>
<th>C18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>9 (53%)</td>
<td>9 (23%)</td>
</tr>
<tr>
<td>Anaerobacter variabilis</td>
<td>9 (27%)</td>
<td>9 (52%)</td>
</tr>
<tr>
<td>Mycobacterium phlei</td>
<td>10 (32%)</td>
<td>9 (7%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organisms with anaerobic mechanism</th>
<th>C18</th>
<th>C20</th>
<th>C22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>11 (19%)</td>
<td>11 (36%)</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus arabinosus</td>
<td>11 (12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonasfluorescens</td>
<td>9 (31%)</td>
<td>11 (12%)</td>
<td></td>
</tr>
<tr>
<td>Rhodopseudomonas spheroides</td>
<td>11 (12%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE III**

**Formation of hexadecenoic acids from palmitic acid in Mycobacterium phlei**

The labeled acids were added to 100 ml of *M. phlei* medium (see Footnote b, Table I) before inoculation.

<table>
<thead>
<tr>
<th>Reaction product</th>
<th>1-C4-palmitic acid</th>
<th>U-C4-palmitoleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>8,000 c.p.m./µg</td>
<td>14,000 c.p.m./µg</td>
</tr>
<tr>
<td>Total activity added</td>
<td>1.4 X 10⁶ c.p.m.</td>
<td>1.4 X 10⁶ c.p.m.</td>
</tr>
<tr>
<td>C4 incorporated into total fatty acids</td>
<td>55,000 c.p.m. = 3.5%</td>
<td>95,000 c.p.m. = 6.6%</td>
</tr>
<tr>
<td>Specific activity of isolated C4-fraction</td>
<td>30 c.p.m./µg</td>
<td>132 c.p.m./µg</td>
</tr>
<tr>
<td>Specific activity of isolated C4-fraction before oxidation</td>
<td>45 c.p.m./µg</td>
<td></td>
</tr>
<tr>
<td>Specific activity of oxidation products</td>
<td>C4-dicarboxylic acid</td>
<td>75 c.p.m./µg²</td>
</tr>
<tr>
<td>C4-dicarboxylic acid</td>
<td>53 c.p.m./µg²</td>
<td></td>
</tr>
</tbody>
</table>

a The 1-C4-palmitic acid, purchased from the New England Nuclear Corporation, was at least 95% pure and contained less than 1% hexadecenoic acid as determined by gas-liquid chromatography.

b Uniformly labeled palmitoleic acid was synthesized by growing yeast in a medium containing 1-C4-sodium acetate. Methylpalmitoleate was isolated from the unsaturated fatty acid fraction by repeated gas chromatography. The ester was hydrolyzed and the free acid added to the *M. phlei* medium.

c The higher specific activity of the oxidation products than of the intact acids shows that there is little randomization of C4 when palmitate is converted to hexadecenoate. The calculated specific activities for the C7- and C9-dicarboxylic acids, assuming no randomization of C4, are 80 and 72 c.p.m. per µg, respectively.

d The increased specific activity of the C4-dicarboxylic acid relative to that of the entire C4-fraction is due to the fact that the Δ² component in the intact fatty acids is essentially inactive and dilutes the specific activity of the C4-fraction.

The lipids of a given organism contain one or more series of two and in some cases of three homologous even-numbered fatty acids. In one of the series, found in several organisms, the double bonds are in all cases located between the 7th and 8th carbon atom counting from the methyl end of the fatty acid molecule (Δ'-C16, Δ²-C16, and Δ²-C16). In at least one organism (C. butyricum) there is evidence for a series of acids having the double bonds between the 9th and 10th carbon atom from the methyl end of the molecule (Δ²-C16 and Δ²-C16). A logical mechanism for the synthesis of these series is a successive addition of C2 units to the carboxyl group of an unsaturated fatty acid of the general structure CH3(CH2)₆CH=CH(CH2)₇-COOH (n = 3, 5, or 7) without reduction or dehydrogenation of the double bond. It has been observed by Hofmann et al. (6) that the cis isomers of the series Δ²-C16, Δ²-C16, and Δ²-C16 have biotin-sparing activity for lactobacilli, substituting for Δ²-octadecenoic acid in this respect (24). Hofmann et al. (6) have suggested that these short chain acids exert their biotin-sparing effect either by being converted to Δ²-octadecenoic acid or by substituting for it metabolically. A detailed mechanism for the synthesis of Δ²-octadecenoic acid and homologous acids by microorganisms has been discussed by Kaneshiro and Marr (9).
Fig. 1. Postulated anaerobic pathway to monounsaturated fatty acids in bacteria. The fatty acids shown in the enclosed area have been isolated from bacterial lipids.

elongation of shorter chain unsaturated acids has recently been proposed in this laboratory (4). It is based on the finding that in C. butyricum, octanoate is elongated to \( \Delta^2\)-C'16 and \( \Delta^3\)-C'18 acids, and decanoate to \( \Delta^2\)-C'16 and \( \Delta^3\)-C'18 acids (3, 4). According to this scheme the pathways to long chain unsaturated and saturated fatty acids branch at the C4 or C6 stage, the medium length acids adding C2 units to form \( \beta\)-hydroxy acids and the latter undergoing \( \beta,\gamma \) elimination of water. Once \( \beta,\gamma \) double bonds are introduced the carbon chain may be extended without reduction of the double bond. A sequence of reactions starting from octanoate by way of \( \beta\)-hydroxydecanoate and \( \beta,\gamma\)-decanoate would afford the series \( \Delta^2\)-C'14, \( \Delta^3\)-C'14, and \( \Delta^4\)-C'16 by addition at the C16 stage of 4, 6, and 8 carbon atoms, respectively. Correspondingly, a process starting from decanoate would account for the \( \Delta^2\)-C'16 and \( \Delta^3\)-C'18 acids found in C. butyricum. Furthermore, the same general process explains the presence of \( \Delta^3\)-C'16 acids in a Streptococcus species (8) and in Clostridium pasteurianum if there is an additional branch-point at the C6 stage yielding \( \beta,\gamma\)-octenoate and thence \( \Delta^4\)-hexadecenoic acid by four C2 additions. Other long chain unsaturated fatty acids which are predictable constituents of bacterial lipids on the basis of this mechanism are \( \Delta^7\)-tetradecenoic acid (from the decanoate branch) and also \( \Delta^3\)-tetradecenoate and \( \Delta^3\)-octadecenoate (hexanoate branch; Fig. 1).

There is evidence, although still incomplete, that the synthesis of all bacterial unsaturated fatty acids by the mechanism shown in Fig. 1 occurs under anaerobic conditions. This is certainly true for the various corynebacteria. Moreover, in E. coli and Lactobacillus arabinosus the fatty acid patterns are identical whether the cells are grown in air or anaerobically, or in the case of Rhodopsseudomonas, anaerobically in the light or aerobically in the dark. It is clear from our findings that the anaerobic mechanism for synthesizing unsaturated fatty acids is employed not only by anaerobes but also by aerobic members of the order Eubacteriales, i.e. there is no relation between the mode of synthesis and the presence or absence of the cytochrome system. Moreover, since the more advanced microorganisms listed in the upper section of Table II and probably all higher organisms employ an oxidative pathway to unsaturated fatty acids, it appears that the distribution of the aerobic and anaerobic mechanism follows evolutionary lines and is not a function of aerobic versus anaerobic modes of existence.

While in plants and animal tissue oleic acid is the most abundant monounsaturated fatty acid, it is in some instances accompanied by significant amounts of \( \Delta^3\)-octadecenoic acid, for example in brain (25, 26) and in certain seed oils (Asclepia syriaca) (27). The origin of \( \Delta^3\)-octadecenoic acid in these systems is not known but a chain elongation of palmitoleate acid seems to be a more likely mechanism than either oxidative desaturation of palmitoleate acid or synthesis by the bacterial pathway involving chain elongation of \( \beta,\gamma\) unsaturated acids.

Our observations on the distribution of the two pathways, which thus far cover only a limited and selected sample, indicate that a given organism employs either the oxidative or the anaerobic pathway, but not both. Yeast, which forms oleate and palmitoleate by oxidative desaturation, fails to synthesize any unsaturated acids in the absence of oxygen (1). Conversely, in C. butyricum (3), E. coli, or lactobacilli there is no desaturation of long chain saturated to the corresponding unsaturated acids. It is therefore tempting to speculate that the adoption of the oxidative pathway for synthesizing unsaturated fatty acids in the course of evolution was of selective advantage, permitting the organism to abandon the more "primitive" and perhaps more complex anaerobic mechanism.

In the present analysis of bacterial lipids, whether from Eubacteriales or mycobacteria, we have failed to observe any of the highly unsaturated fatty acids, even in traces. Polyunsaturated fatty acids occur in fungi, plants, and animal tissues but the literature does not mention their presence in bacterial sources (5). It is known that the conversion of monounsaturated fatty acids requires oxygen (28) and therefore it is not surprising that these compounds are absent in organisms which synthesize unsaturated fatty acids anaerobically. On the other hand, the presence of the aerobic route as in mycobacteria, while a prerequisite, is apparently not a sufficient condition for the synthesis of polyunsaturated acids, suggesting that the appearance of these compounds was a still later evolutionary event.

**SUMMARY**

1. The unsaturated fatty acids isolated from the total lipids of various microorganisms have been analyzed by oxidative degradation and gas-liquid chromatography. \( \Delta^3\)-Octadeenoic and

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4 Recent experiments by P. Baronowsky in this laboratory have demonstrated an elongation of \( \beta,\gamma\) cis-decanoate to long chain unsaturated acids by growing cultures of C. butyricum.

5 P. Baronowsky, unpublished experiments.
palmitoleic acids are the major unsaturated acids in all the cubacteria examined. Some of these organisms contain in addition \( \Delta^5 \)-C\(_{16} \), \( \Delta^11 \)-C\(_{16} \), \( \Delta^9 \)-C\(_{16} \) acids and the previously unknown \( \Delta^9 \)-tetradecenoic acid.

2. The lipids of *Mycobacterium phlei* have been shown to contain cis-\( \Delta^10 \)-hexadecenoic acid in addition to smaller amounts of palmitic acid. Palmitoleic acid is not a precursor of the \( \Delta^10 \) isomer.

3. The correlation between the double bond structure of microbial fatty acids and the type of synthetic mechanism employed by the organism is discussed.

REFERENCES


