Unsaturated Fatty Acid Biosynthesis in *Tetrahymena*

EVIDENCE FOR TWO PATHWAYS

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The ciliate *Tetrahymena pyriformis* synthesizes a wide variety of saturated and unsaturated fatty acids. Variations in growth temperature or the addition of sterols such as cholesterol or ergosterol alter the proportions of a number of unsaturated fatty acids. The pattern of substitution is complex when examined on the basis of individual fatty acids. A straightforward explanation is possible, however, if biosynthetic groupings are considered. Stearic acid gives rise to oleic, linoleic, and γ-linolenic acids as shown by others. The environmental parameters influence the amounts of the unsaturated derivatives of stearic acid as a group and result in an increase or decrease in all members of this biosynthetic family in a fashion that depends on a particular set of conditions. The replacement of the stearate derivatives by unsaturated components that are derived from palmitic acid has been demonstrated.

The addition of radiolabeled palmitic, palmitoleic, or stearic acids to the cells when coupled with radioisotope distribution measurements, isolation, and characterization of the acids, and the refeeding of key intermediates substantiates a second biosynthetic scheme for the synthesis of unsaturated acids in this ciliate. This novel pathway yields an unusual fatty acid, 18:2 Δ⁶,¹¹, as a major terminal product.

Palmitic acid is the precursor for the members of both sequences. Palmitoleic acid can be desaturated further to produce two hexadecadienoates and a hexadecatrienioate. Further, palmitoleic acid (16:1 Δ⁹) is elongated to cis-vaccenic acid (18:1 Δ¹¹) which is then desaturated to 18:2 Δ⁶,¹¹. Linoleic and γ-linolenic acids were essentially unlabeled when [¹⁴C]palmitoleic acid was provided. Refeeding radiolabeled 18:2 Δ⁶,¹¹ to the cells revealed the extensive incorporation of this acid into the polar lipids and the absence of additional metabolites. [¹⁴C]Stearic acid addition to the cells results in extensive labeling of linoleic and γ-linolenic acids, but not of 18:2 Δ⁶,¹¹. These observations confirm the existence of alternative and separate pathways for the synthesis of unsaturated fatty acids.

Unsaturated acids in this organism arise from stearate. The sphingolipids contain even normal and odd iso saturated acids and their α-hydroxy analogues (5). The cellular fatty acyl composition is subject to marked alterations in response to temperature shifts (11-16) and to supplementation with long chain acids (7, 17, 18), short chain acids (3, 4, 6, 19), or sterols (5, 20).

This ciliate contains no true sterols; however, the sterol function(s) seem to be served by the pentacyclic triterpenoid alcohol, tetrahymanol (for review see Refs. 2 and 21). The addition of a sterol, such as ergosterol, to the culture fluid results in curtailed synthesis of tetrahymanol and the replacement of the native alcohol in the limiting membrane by the exogenous agent (22-24). Either a downward shift in temperature (16) or supplementation with ergosterol (5, 20) decreases the cellular content of 18:0, 18:1 Δ⁹, 18:2 Δ⁶,¹², and 18:3 Δ⁶,⁹,¹² fatty acids, all products of the major endogenous biosynthetic pathway. A concomitant increase in palmitoleic, two isomers of 18:2, 18:3, 18:2 Δ⁶,¹¹, and 20:1 Δ¹⁰ fatty acids in membrane lipids occurs (5, 16).
The reciprocal increase and decrease in the two groups of fatty acids could be explained by assuming that the conditions employed led to the preferential accumulation of the individual acids. The basis for such a selection, however, is difficult to envision due to the variety of acids involved. An alternative interpretation is based on the observation that all of the fatty acids that decrease in amount belong to a common biosynthetic series. Thus, it was reasonable to anticipate that all of the acids that were elevated at low temperatures or in the presence of ergosterol also belong to a common and unique pathway. In this latter case the environmental factors could lead to the stimulation of one synthetic sequence and/or to the inhibition of a second. This, in turn, would result in the elevation of the products of one series at the expense of the other.

This paper provides evidence for a novel synthetic scheme in _Tetrahymena_ that emanates from palmitoleic acid and leads to more highly unsaturated 16-carbon derivatives which include two isomers of hexadecenoic and a hexadecatrienoic acid. In addition, elongation of palmitoleate occurs to yield cis-vaccenate (18:1 Δ⁵) which is desaturated to produce 18:2 Δ⁴,⁵.

**METHODS**

Stock cultures of _Tetrahymena pyriformis_ W in the stationary phase of growth were used to seed low form _Ehrlichmyer_ flasks which contained 500 ml of 2% filtered proteose peptone (Difco), yeast extract (Difco), 90 μM iron-EDTA complex (25), and, in specific cases, a supplement of one of three fatty acids. Methyl palmitate, palmitoleate, and stearate were obtained from Applied Science Laboratories and checked for purity both by argentation and by gas-liquid chromatography. Cell numbers were estimated with a Coulter particle counter equipped with a 200-μm aperture.

The ciliates were incubated at 28.5 ± 0.5°C with 20 μM [1-¹⁴C]palmitoleic or [1-¹⁴C]stearic acid (specific activity, 6.5 mCi/mmol), claimed in early stationary stage of growth, lyophilized, and extracted with chloroform/methanol, 2:1 (v/v) as previously described (24). Supplementation experiments with 1 μCi of [1-¹⁴C]palmitic acid (specific activity, 55.7 mCi/mmol) differed only in that the fatty acid was introduced 5 h prior to cell harvest. The total lipid extract was freed from nonlipid contaminants by Sephadex chromatography. Cell numbers were estimated with a Coulter particle counter equipped with a 200-μm aperture.

Resolution of the total lipid fraction into neutral and polar lipid extract was completed by argentation chromatography (28). Of the fatty acid methyl esters, 54 mg were eluted with increasing proportions of benzene in petroleum ether (b.p. 40-60°C) from a column packed with 5 g of Unisil impregnated with 20% AgNO₃ (w/w). At least two 50-ml portions were collected with each solvent mixture. All fractions were analyzed by gas-liquid chromatography (Biomedical Gas Chromatograph, F & M model 4012) using a 6-foot column packed with 80 to 100 mesh Gas- Chrom-P coated with 12% diethylene glycol succinate at 170°C and by liquid scintillation spectrometry. The fatty acid methyl esters were identified on the basis of elution patterns from the columns, gas-liquid chromatographic retention times relative to methyl stearate, hydrogenation, oxidation, and mass spectrometry as described earlier (5). Quantitation was performed by weighing the individual peaks. Fractions comprised of more than one fatty acid methyl ester were rechromatographed to yield at complete separation as possible.

All samples were placed on 1-g Unisil columns and eluted with chloroform to remove the contaminating silver. This was necessary to reduce quenching in the radioactive determinations. No loss of fatty acid methyl esters occurred during this step. The specific activity for each fraction was calculated from data obtained from the radioactivity measurements and quantitative gas-liquid chromatographic analyses of pure samples. In cases where incomplete resolution of fatty acid methyl esters was encountered, additional evidence for compound identity and localization of radioactivity was gathered by thin layer chromatography followed by autoradiography (3, 23). Plates coated with 76% AgNO₃ (w/w) in Silica Gel G were developed with hexane/petroleum ether, 1/1 (v/v), and visualized with photographic acid. Reversed phase chromatograms were obtained on plates covered with 0.3 mm of Silica Gel G treated with dichloromethylene and processed with 10% H₂O in MeOH saturated with AgNO₃ (29). These procedures resolved the monoenoas, especially the methyl palmitoleate and octadecenoenoate components, as well as the C₁₈₋ and C₁₆₋ dienoic acids. These techniques also provided further supportive evidence for the distribution of radioactivity in mixtures of 18:2 Δ⁴,⁵ and 18:3 Δ⁵,⁶,⁷.

Radiolabeled 18:2 Δ⁴,⁵ methyl ester was isolated from _Tetrahymena_ lipids by the above procedure in a highly purified form (>99%) as judged by gas-liquid chromatography, mass spectrometry, thin layer chromatography, and maintenance of a constant specific activity (1.7 × 10⁶ cpm/mg). The free fatty acid was generated (8.1 × 10⁵ cpm), dissolved in ethanol, and introduced into 500 ml of culture fluid. Cells were added and incubated at 38.5°C for 21 h. The fatty acids were isolated, identified, and quantitated as described above.

**RESULTS**

A concentration of 20 μM palmitoleic, stearic, or palmitic acid did not inhibit the growth of the cells when the initial inoculum was approximately 3.8 × 10⁵ cells/500 ml of culture fluid with a pH value of 6.5. No consistent differences in the dry weight or lipid content were observed between the controls and experimentals (Table I). An acid concentration of 40 μM resulted in immediate cell death of most of the population (>90%); however, the surviving cells proved viable and eventually reached a density of approximately 50% that of control cultures. Culture fluid with 60 μM concentrations of any of the three acids led to immediate lysis of all cells in the inoculum (30).

**Palmitoleic Acid Supplementation**—Cells grown with [1-¹⁴C]palmitoleic acid incorporated 67% (3.32 × 10⁷ cpm) of the added radioactivity into the purified lipid fraction (Table I). Resolution of this total lipid fraction into neutral and polar lipids gave a distribution by weight of 16% in the former and 84% in the latter, while 57% of the radioactivity was recovered in the polar materials. The neutral lipids were not examined further.

Alkaline methanolysis of the polar lipids was followed by the isolation of the fatty acid methyl esters by column chromatography. The fatty acid methyl esters were resolved by argentation chromatography as described.

The saturated fatty acids contained a low level of radioactivity (2.5%). Rechromatography of the saturates did not alter this value. It is not known if the saturated acids arise by reduction of the monoene or by β oxidation of the palmitoleic acid and reincorporation of the label (2, 31).

The monoenoic methyl esters were examined by reversed thin layer argentation chromatography and two unlabelled components were detected. The RF values compared to standards were in agreement with the assignments of methyl palmitoleate and methyl octadecenoate. We were unable to resolve cis-vaccenate and oleic acids satisfactorily with this procedure. Gas-liquid chromatography on a 50-foot diethylene glycol succinate support-coated open tubular column (118°C; helium, 5 ml/min) gave peak separation of two components in the 18:1 region, one of which cochromatographed with methyl cis-vaccenate and the other with methyl oleate. Oxidation of the monoenoic methyl ester fraction, methylation of the dicarboxylic acid oxidation products, and subsequent analysis of the dimethyl dicarboxylic acid fragments by gas-liquid chromatography revealed a C₁₈ dimethyl component which cochromatographed with the dimethyl ester of undecenoic acid (Aldrich Chemical Co., Inc.). In addition, a C₁₆ dimethyl ester
Comparison of dry weight, lipid composition, and distribution of radioactivity in Tetrahymena grown with palmitic, palmitoleic, or stearic acid

<table>
<thead>
<tr>
<th>Palmitate</th>
<th>Palmitoleate</th>
<th>Stearate</th>
<th>lipids</th>
<th>msp dry wt</th>
</tr>
</thead>
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<tr>
<td>219</td>
<td>207</td>
<td>206</td>
<td>141</td>
<td>150</td>
</tr>
<tr>
<td>58% (1.12 × 106)</td>
<td>67% (3.22 × 106)</td>
<td>80% (6.04 × 106)</td>
<td>88% (2.88 × 106)</td>
<td>81% (2.23 × 106)</td>
</tr>
</tbody>
</table>

* TLS, total lipids after Sephadex purification.
* Percentage of total radioactivity added to the culture that was recovered in the cellular lipids; counts per min in lipid fraction is shown in parentheses.
* Percentage of radioactivity in the lipid fraction found in the polar lipids; counts per min in polar lipids is shown in parentheses.

which cochromatographed with dimethyladipic acid (Eastman Kodak, Inc.) was noted. The latter would be expected to come primarily from palmitoleic and oleic acids while the former would arise from a Δ11 monoene such as cis-vaccenate. Thus, strong evidence exists for the occurrence of cis-vaccenate as well as oleic acid in the methyl octadecamonoenoic acids of the supplemented cells. Both C16 components were detected also in unsupplemented cells.

Methyl linoleate (18:2 Δ12) and γ-linolenate were isolated by column argentation chromatography and after rechromatography were found to contain 42.8% of the total mass but only 0.5% of the total radioactivity associated with the fatty acid methyl esters (Table II). Difficulty was encountered in our attempts to obtain complete separation of two methyl hexadecadienoates from methyl linoleate. A column fraction (3.22 × 105 cpm) consisting of 98.4% 18:2 Δ12 and less than 0.3% of the hexadecadienoic methyl esters showed no detectable radioactivity associated with the linoleic ester after thin layer reversed phase argentation chromatography and autoradiography. A second fraction composed of 64.4% methyl linoleate and 31.5% of the 16:2 isomers treated as above gave a single spot on autoradiograms that corresponded to the hexadecadienoic methyl esters. Similarly, a fraction consisting of 78.6% γ-linolenic acid and 21.3% 18:2 Δ11 showed only a single labeled component that cochromatographed with methyl oleate. The methyl ester of 18:2 Δ11 was found to be heavily labeled (1.7 × 106 cpm/mg) and to contain approximately one-quarter of the total radioactivity in the fatty acid methyl ester fraction (Table II).

Another highly labeled component emerged from the argentation column following the complete elution of methyl γ-linolenate. This material (1.9 × 106 cpm/mg) contained 9.4% of the radioactivity associated with the fatty acid methyl esters. The point of emergence of the unknown from the argentation columns, the high specific activity, and the gas-liquid chromatographic retention time of 1.12 relative to methyl stearate suggested a hexadecatrienoic methyl ester. Catalytic hydrogenation yielded a product that was identical with methyl palmitate by gas-liquid chromatographic analysis. Gas chromatography-mass spectrometry verified the assignment as the methyl ester of 16:3 (M+ 264). The double bond positions were not determined.

An incubation with 8.1 × 105 cpm of the purified 18:2 Δ11 was carried out to determine if the material was incorporated into the polar lipids and to examine if further metabolic alteration occurred. A total of 7.16 × 105 cpm (88%) was recovered in the fatty acid methyl esters derived from the polar lipid materials. The radioactivity was associated with fractions containing the octadecadienoic acid (Fig. 1). There was no evidence for the presence of additional radiolabeled metabolites.

<table>
<thead>
<tr>
<th>Palmitate</th>
<th>Palmitoleate</th>
<th>Stearate</th>
<th>% cpm</th>
<th>% weight</th>
<th>% cpm</th>
<th>% weight</th>
<th>% cpm</th>
<th>% weight</th>
<th>% cpm</th>
<th>% weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.7</td>
<td>32.0</td>
<td>88.0</td>
<td>13.9</td>
<td>23.7</td>
<td>5.6</td>
<td>25.6</td>
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<td>45.8</td>
<td>3.6</td>
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<td>30.7</td>
<td>21.3</td>
<td>86.4</td>
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<td>38.9</td>
<td>1.7</td>
<td>32.2</td>
<td>2.0</td>
<td>58.7</td>
<td>2.2</td>
<td>56.6</td>
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* In the fatty acid methyl esters obtained from palmitoleic acid incubations, the isomers of 16:2 contained 2.6 × 106 cpm or 14.4% of the radiolabel; 16:3 had 1.9 × 106 or 9.4% of the total radioactivity.
* All percentages are based on total weight and radioactivity present in the original mixture of fatty acid methyl esters.

**Stearic Acid Supplementation**—Stearic acid was incorporated readily by the ciliates (Table I). Surprisingly little (2.4%) was found in the saturated fatty acids derived from the polar lipids. Reversed phase chromatography followed by autoradiography revealed one labeled component that cochromatographed with methyl stearate. A low level of radioactivity was found in the monoenoic fraction. The labeled material cochromatographed with methyl oleate.

Three polyunsaturated acids, 18:2 Δ12, 18:2 Δ11, and 18:3 Δ9,12, were isolated by column argentation chromatography to a purity of >95% as determined by quantitative gas-liquid chromatography. The linoleic and γ-linolenic acids were heavily labeled (specific activity: 8.0 × 106 and 1.10 × 106 cpm/mg, respectively) and contained >95% of the radioactivity associated with the fatty acid methyl esters derived from the polar lipids (Table II). The second dienoic component, 18:2 Δ11, contained less than 0.1% of the total counts.

**Palmitic Acid Supplementation**—The data from a pulse-label experiment with palmitic acid carried out as described under "Methods" are shown in Tables I and II. Samples of three polyunsaturates (18:2 Δ11, 18:2 Δ12, 18:3 Δ9,12) ob-
tained by argentation chromatography proved to be of high purity (> 92%) as determined by gas-liquid chromatography. The percentage of total counts associated with the fatty acid methyl esters of the polar lipids are listed in Table II. The methyl esters of γ-linolenic and of 18:2 \( \Delta^6,11 \) fatty acids occur in a ratio of approximately 8:1:3 in this ciliate. The 18:2 \( \Delta^9,12 \), 18:2 \( \Delta^9,11 \), and 20:1 \( \Delta^{13} \) fatty acids are found which presumably arise from oleate (5). A third unusual octadecadienoate isomer 18:2 \( \Delta^8,11 \) also exists in this ciliate. The 18:2 \( \Delta^9,11 \) isomer appears to have been misidentified as 18:2 \( \Delta^9,12 \) vaccenate (13, 32, 33) and 20:1 \( \Delta^{13} \) are found which presumably differ from that of bacteria (35). Thus, there is reasonable evidence to propose the sequence: palmitoleate (16:1 \( \Delta^9 \)) \( \rightarrow \) cis-vaccenate (18:1 \( \Delta^{11} \)) \( \rightarrow \) cilienic acid (18:2 \( \Delta^{11,14} \)). This octadecadienoic acid, a major component of the glycerophospholipids, does not appear to be reduced or desaturated further. Cis-vaccenate also may be elongated to 20:1 \( \Delta^{13} \) especially when the cells are grown at low temperatures (16).

A pathway for direct palmitoleate desaturation is present, although only minor amounts of the products, two isomers of hexadecadienoic and hexadecatetraenoic acid, are found even in palmitoleic acid-enriched cultures. The occurrence of the dienes and the triene suggests a sequence similar to that of oleate desaturation, namely 16:1 \( \Delta^9 \) \( \rightarrow \) 16:2 \( \Delta^9,12 \) or 16:2 \( \Delta^8,9 \) \( \rightarrow \) 18:3 \( \Delta^8,9,12 \). The elution pattern of these palmitoleate derivatives from silver nitrate-Unisil columns and the retention times of the parent methyl ester on 12% diethylene glycol succinate columns are consistent with this formulation. The desaturation sequence is analogous to that seen with stearic acid and suggests the same desaturase may be operative on the C-16 and C-18 acids with the major difference being a greater affinity for the longer chain acids. A summary of the fatty acid metabolic pathways in Tetrahymena is presented in Fig. 2.

The direct incorporation of a high percentage of labeled 16:2 \( \Delta^{11} \) into the polar lipids and the recovery of nearly 90% of the label in unaltered form will allow the measurement of the fatty acyl turnover rate for this acid. Since 18:2 \( \Delta^{11,14} \) is associated primarily with 1,2-diacyl-sn-glycero-3-(2-aminoethyl)phosphonate and with 1-alkyl-2-acyl-sn-glycero-3-(2-aminoethyl)phosphonate (5) which are enriched in the cellular-limiting or ciliary membranes, it should be possible to estimate a turnover value for an important component of this organelle with considerable precision.

Replacement of the naturally occurring pentacyclic triterpenoid alcohol, tetrahymanol, by ergosterol leads to a readjustment in the cellular lipids that is characterized by an increase in the products of the palmitoleic acid pathway (5).
An even greater impact on the fatty acyl composition is seen with a shift in environmental temperature. As the temperature is lowered, more of the palmitoleic acid derivatives are seen in the polar lipids at the expense of linoleic and γ-linolenic acid. Either of these sets of data may be explained on the basis of an alteration in fatty acid biosynthesis which leads to increased (decreased) availability of a given series of acids for incorporation into polar lipids, on the differential accumulation of fatty acids into polar lipids governed by acyl transferases, or on a change of the turnover rates of specific fatty acyl moieties in the polar lipids. The information now available will allow these three alternatives to be examined in a systematic fashion.

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REFERENCES


Unsaturated fatty acid biosynthesis in Tetrahymena. Evidence for two pathways.
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