Environmentally-Induced Changes in the Fatty Acids of Chlorella1, 2

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Abstract. Qualitative and quantitative analyses were made of the major fatty acids of Chlorella fusca, Shihira and Krauss, in both autotrophic and heterotrophic culture. Cells grown heterotrophically were subjected to CO₂ concentrations as great as 40% in-air.

The major fatty acids of autotrophic cells grown under a 1% environmental concentration of CO₂, in order of concentration were 16:0, 18:3, 16:4, 18:2, 18:1, and 16:3. The analysis of heterotrophic cells at 1% CO₂, by comparison, indicated a complete absence of 16:4, reduced amounts of 18:3, and increased amounts of the other major acids.

An increase in the environmental concentration of CO₂ (from 1%–30%) over the heterotrophic cultures produced a 40% increase in total lipids and nearly a 50% increase in fatty acids. Palmitic acid (16:0) and 18:1 largely account for the fatty acid change by increasing from 12 mg to 33 mg/g dry wt and 8 mg to 17 mg/g dry wt respectively. Possible explanations for this enhanced synthesis of fatty acids are suggested.

Carbon dioxide-enriched air has long been employed as a carbon source in the growth of unicellular, photosynthesizing algae, especially for physiological experimentation. However, little effort has been directed toward analysis of the changes which unnatural concentrations of CO₂ might effect on algal metabolism (3, 7, 8, 13). It is well-established that the lipid content, especially the fatty acids, of an organism such as Chlorella can be readily and drastically varied experimentally by changing the physical and/or chemical nature of the environment. Spoehr and Milner, (15), among others, confirmed that the assimilation of carbon, either as CO₂ or as organic carbon, probably continues after some environmental factor (i.e., limiting nitrogen) has brought cell division to a halt. That CO₂ has a direct inhibitory effect on cell division of Chlorella has been established by Sorokin (14). Production of new protoplasm and growth of the culture may be highly sensitive to environmental stress, whereas carbon continues to be assimilated and can only accumulate as reserve products such as triglycerides.

Although a number of workers have investigated the fatty acids of Chlorella (1, 2, 3, 5, 9, 10, 11, 17), to date there has been no investigation into the effects of a wide range of environmental concentrations of CO₂ on the fatty acids of any organism.

The purposes of this study then, were first to extract, identify, and quantitatively analyze the fatty acids of autotrophic and heterotrophic cells of Chlorella fusca, grown under a 1% CO₂-in-air mixture—a standard environmental condition in our laboratory. Secondly, changes in total lipids and fatty acids as brought about by an increase in the environmental concentration of CO₂ were investigated. These experiments constitute the initial steps into an investigation of why and how Chlorella accumulates fatty acids.

Materials and Methods

Culture Conditions. Chlorella fusca, Shihira and Krauss (12), Indiana Algal Culture Collection No. 343, was cultured in sterile media (16) at 29° in specially prepared culture tubes measuring 43 cm by 4.5 cm. These had a conical bottom and were fitted with a bubbling tube held in place with a silicon rubber stopper.

Autotrophic cells were grown at a light intensity of 1650 ft-c provided by dual banks of 40-watt G.E. cool-white lamps and measured by a Weston Illumination Meter at the surface of the culture vessels. A 1% CO₂-in-air mixture was bubbled through the medium as the carbon source and as a convenient method of keeping the cells in suspension. Cells grown heterotrophically were in complete darkness, 0.86% glucose was the carbon source, and the

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CO₂-in-air was supplied at 1 %, 5 %, 10 %, 20 %, 30 %, or 40 %. The 1 % mixture was prepared in our laboratory; the others were supplied from Air Products and Chemicals, Ltd.

**Extraction of the Lipid and Isolation of the Fatty Acids.** Cells were harvested by centrifugation at 1000g for 10 min and immediately heated with dimethyl sulfoxide for 15 to 20 min at 60 to 65°. An equal volume of methanol was added, heating was continued for 5 min. followed by extraction with chloroform-methanol, 2:1 (v/v), in a soxhlet extractor overnight. The entire extract was taken to dryness in vacuo, redissolved in chloroform, filtered into a weighed beaker, and evaporated to dryness under nitrogen. The dry residue was weighed and is reported as total lipid.

The total lipid was saponified with 5 % KOH in 70 % ethanol for 1 and one-half to 2 hr at approximately 70°. The non-saponifiable portion was removed from the saponified aqueous sample by partitioning against anhydrous ether in a liquid/liquid extractor overnight. The remaining saponified material was treated with 6 N HCl and again partitioned against ether overnight. The fatty acids from the ether fraction were methylated with 3 to 4 ml of boron trifluoride-methanol in a boiling water bath for 5 min. Ten ml of water was added, the sample cooled, and washed with n-hexane 4 to 5 times. The hexane extracts were combined and the sample taken to dryness under nitrogen. This yielded fatty acid methyl esters for analysis by gas-liquid chromatography (GLC).

**Fatty Acid Analysis.** All gas chromatographic analyses were made with a Chromalab Model A-110 gas chromatograph (GlowaⅠ Corp.) equipped with an argon β-ionization detector. This was coupled with a Honeywell 12-inch recorder fitted with a disc integrator. Coiled glass columns (6 feet by 3.4 mm, i.d.) were packed with Gas-Chrom P, 60 to 80 mesh (Applied Science Lab) coated with 15 % diethylene glycol succinate (DEGS), and 2 % H₃PO₄. All columns were conditioned before use at 190° for at least 24 hr.

The argon carrier gas was maintained at a pressure of 15 or 20 psi and the column temperature was 158 to 165°. The fatty acids were dissolved in n-hexane and injected into the column with a Hamilton 10 μl syringe.

An initial qualitative analysis was made by injection of a standard mixture of fatty acids either before or after the sample. Quantitative analyses were performed with the use of the disc integrator and a C 20:0 quantitative standard containing either 2 or 4 μg/μl of solvent.

Thin-layer chromatography (TLC) was employed to separate the fatty acids into groups according to the number of double bonds. All plates were prepared of 12 % AgNO₃ in Silica Gel G which was applied in a 1 mm thick layer onto 20 cm by 20 cm glass plates. These were activated at 110° for 70 min and the fatty acids applied immediately upon cooling. Development was in 20 % ether in n-hexane to a height of 15 cm at which time the plates were air-dried and sprayed with a 0.2 % solution of dichlorofluorescein in 95 % ethanol. Upon examination under ultraviolet radiation, 4 distinct bands were observed with approximate RF values of 0.7, 0.5, 0.3, and 0.1 and corresponding to the saturated, monounsaturated, diunsaturated, and polyunsaturated fatty acids respectively. The fatty acids were eluted from the silica gel by washing several times with ether. A rotary flash evaporator was used to remove the ether and the dry sample was redissolved in n-hexane and rechromatographed on GLC. By this procedure a quantitative recovery of greater than 95 % could always be made, as shown by comparative GLC patterns before and after TLC.

Conversion of unsaturated to saturated fatty acids further substantiated the identification of the various peaks. This was accomplished by adding platinum oxide to the fatty acid sample in n-hexane and bubbling with H₂ for 8 to 10 min.

**Results and Discussion**

**The Fatty Acids of Autotrophic and Heterotrophic Cells.** The major fatty acids of autotrophic cells grown at 1 % CO₂ were shown to be 16:0, 16:3, 16:4, 18:1, 18:2, and 18:3 (table I). There are minor amounts of 16:1, 16:2, and 18:0, as well as minor amounts of other fatty acids which were not positively identified. These are indicated as A, B,

<p>| Table I. The Fatty Acid Composition of Autotrophic and Heterotrophic Cultures of Chlorella fusca. Both Bubbled Continuously With a 1 % CO₂-in-air Mixture |
|---------------------------------|---|---|---|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>16:0</th>
<th>16:1</th>
<th>16:2</th>
<th>18:0</th>
<th>18:3</th>
<th>18:1</th>
<th>18:4</th>
<th>18:2</th>
<th>18:3</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autotrophic cultures</td>
<td>5.3</td>
<td>0.4</td>
<td>38.1</td>
<td>0.6</td>
<td>1.1</td>
<td>0.8</td>
<td>4.3</td>
<td>13.7</td>
<td>t¹</td>
<td>4.9</td>
<td>6.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Heterotrophic cultures</td>
<td>0.8</td>
<td>t</td>
<td>40.8</td>
<td>2.0</td>
<td>t</td>
<td>6.5</td>
<td>8.0</td>
<td>0.0</td>
<td>t</td>
<td>25.6</td>
<td>13.1</td>
<td>t</td>
</tr>
</tbody>
</table>

¹ t indicates trace amounts (less than 0.2 % of the total fatty acids).
C. D, and E in table I and are maintained in the same order as their emergence from the GLC column. Hydrogenation of the total sample revealed that all compounds had 14, 16, or 18 carbon atoms in their structures. Palmitic acid was present in the largest amount (38%) with relatively large quantities in 16:4 and 18:3.

In comparing data from heterotrophic cells with those for the autotrophic cells (table I), there was a complete loss of 16:4, a large decrease in the relative amount of 18:3, and an increase in the relative amount of 18:1. It was also found that 16:2, 16:3, and 18:2 increased, while compound A decreased in relative amount. Other acids were almost exactly as in the autotrophic cells.

The Effects of High Concentrations of Carbon Dioxide. When cultures were exposed to 40% CO₂-in-air, they showed no increase in either optical density or dry weight. The environmental stress did not cause death, but growth was not measurable over periods as long as 3 weeks. The cultures under 30% CO₂ grew only slightly and became yellow-green to pale grey-green in color. Chloroplasts were more irregular than usual and the cytoplasm very granular in appearance. The average cell size increased and delay of daughter-cell release was marked. These conditions were also noted in cells grown at 20% CO₂ but to a much reduced degree. The growth and morphological characteristics of the 5% and 10% cultures were practically indistinguishable from the controls (1%).

As the cells were grown at increasingly higher concentrations of CO₂, there was an increase in the total amount of lipid synthesized (table II). The increased total lipid production compared to the 1% cultures ranged from 16% (5% cultures) to 40% (40% cultures). Inasmuch as the extraction removed all pigments from the cell as well as lipids of all kinds, it was not possible to determine whether part or all of the lipids increased in amount. Chlorophyll accounts for a large portion of the lipid from the 1% and 5% cultures, but chlorophyll concentration decreases rapidly as the lipid content increases (4). Thus these figures may not indicate the true magnitude of the lipid change.

However, fatty acids, as a percent of dry weight (table II), also showed an increase. In this case, the amount of fatty acid produced at 30% CO₂ is nearly double that produced at 1% CO₂. The low percentage of fatty acid found in cells cultured at 40% CO₂ is presumably attributable to the fact that these cells were not growing and were in fact consuming fatty acids in respiratory pathways faster than they were being produced.

Fatty acids, as percentages of the total lipid, are shown in table II. This reveals a marked decrease in the fatty acids from 5% and 10% cultures, but an increase in the fatty acids of the 20% and 30% cultures.

Qualitatively the fatty acids of heterotrophic cells grown at 5%, 10%, 20%, 30%, and 40% CO₂-in-air did not change from 1% CO₂; however, a significant quantitative change was noted in the relative amount of 16:0 which increased from 41% to 51% of the total fatty acids as the percentage of CO₂ was increased from 1% to 20% (table III). This was accompanied by a relative increase in 16:3 from 8% to 16% (at 10% CO₂), and a decrease in 18:1 from 26% to 14% (at 20% CO₂). The relative amounts of the other fatty acids did not

Table III. The Change in Relative and Absolute Amounts of Fatty Acids of Heterotrophic Cultures of Chlorella fusca, as a Function of the Environmental Concentration of CO₂

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1%</th>
<th>1%</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
<td>2%</td>
<td>1%</td>
<td>2%</td>
<td>1%</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td>16:0</td>
<td>41.1</td>
<td>11.6</td>
<td>40.0</td>
<td>10.0</td>
<td>47.4</td>
<td>8.3</td>
<td>50.9</td>
</tr>
<tr>
<td>16:2</td>
<td>6.5</td>
<td>2.1</td>
<td>5.8</td>
<td>1.6</td>
<td>4.3</td>
<td>1.7</td>
<td>5.0</td>
</tr>
<tr>
<td>16:3</td>
<td>8.0</td>
<td>3.1</td>
<td>15.7</td>
<td>5.1</td>
<td>16.1</td>
<td>4.8</td>
<td>7.3</td>
</tr>
<tr>
<td>18:1</td>
<td>25.6</td>
<td>8.6</td>
<td>17.2</td>
<td>5.3</td>
<td>12.8</td>
<td>3.2</td>
<td>13.6</td>
</tr>
<tr>
<td>18:2</td>
<td>11.3</td>
<td>6.5</td>
<td>10.2</td>
<td>5.9</td>
<td>11.2</td>
<td>4.8</td>
<td>10.9</td>
</tr>
<tr>
<td>18:3</td>
<td>4.8</td>
<td>2.3</td>
<td>5.0</td>
<td>2.7</td>
<td>5.6</td>
<td>2.3</td>
<td>6.3</td>
</tr>
</tbody>
</table>

1 Relative amounts of fatty acids expressed as a percent of the total.
2 Absolute amounts of fatty acids expressed as mg/g dry weight.
change significantly with increase in the environmental concentration of CO₂.

Table III also shows the fatty acids as milligram per gram dry weight of cells. These data indicate that 16:0, 18:1, and possibly 18:2, are the fatty acids which account for the increase in amounts of total acids as indicated in table II. The absolute amount of 16:3 is actually decreasing. These results are consistent with the results of previous workers; i.e., as the concentration of lipid increases the degree of unsaturation decreases.

There seems to be little doubt that fatty acids are synthesized under conditions of high concentrations of atmospheric CO₂, and, in fact, their rates of synthesis may be even higher than under "standard" (i.e. 1% CO₂-in-air) carbon dioxide concentrations. If higher CO₂ concentrations reduce the normal formation of acetyl CoA from pyruvic acid as suggested by Nielsen (6), then possible alternative explanations for the enhanced synthesis of fatty acids could be: (a) that the production of malonyl CoA from acetyl CoA is the rate limiting step in fatty acid biosynthesis, (b) that some other source of large amounts of acetyl CoA is available or, (c) that there is some other pathway for fatty acid biosynthesis, not involving acetyl CoA, than that observed in the various plant species investigated. Further investigation in this area is currently underway.

Literature Cited