Short Communication

Heterotrophic Carbon Dioxide Fixation Products of *Euglena*

EFFECTS OF AMMONIUM

Jennifer G. Peak and Meyrick J. Peak  
*Department of Zoology, Rhodes University, Grahamstown 6140 South Africa*

Irwin P. Ting  
*Department of Botany and Plant Sciences, University of California, Riverside, California 92521*

ABSTRACT

The metabolic products of heterotrophic (dark) CO₂ fixation by *Euglena gracilis* Klebs strain Z Pringsheim were separated and identified. They consisted of amino acids, phosphorylated compounds, tricarboxylic acid cycle intermediates, and nucleotides. Exposure of the cells to NH₄⁺ after a period of NH₄⁺ deprivation stimulated heterotrophic CO₂ fixation almost 4-fold, modifying the spectrum of the fixation products. In particular, the NH₄⁺-treatment stimulated fixation of CO₂ into glutamine, glycine, alanine, and serine.

Heterotrophic CO₂ fixation by *Euglena* was reviewed by Levedahl (7). Although the exact mechanism of the fixation is still unknown, it was shown for *Euglena gracilis* strain Z that dark fixation varies with phase of batch culture, growth, and mode of nutrition in a manner consistent with the possibility that a major function of the dark CO₂ fixation is anaplerotic replenishment of carbohydrate reserves, drained during growth (12). It was also demonstrated that addition of NH₄⁺ stimulated CO₂ fixation in cells previously deprived of this ion (13). This regulatory effect of NH₄⁺ was observed whether the cells were grown phototrophically, or organotrophically on glucose and also occurred in a permanently bleached strain, *E. gracilis* Z SB3.

The range of products of dark CO₂ fixation in *Euglena* differed from that found in other organisms (8). In addition to the usual products of heterotrophic CO₂ fixation such as malate, citrate, and aspartate, in *Euglena* many of the products of photosynthesis such as sugar monophosphates were also present. For this reason it was proposed that *Euglena* may fix CO₂ heterotrophically by two routes, one leading to sugar phosphates by way of P-gluconate, and the other leading into the tricarboxylic acid cycle (10). The exact pathways of dark CO₂ fixation in *Euglena* are yet to be elucidated, and little further work has been reported concerning the products of dark CO₂ fixation. The stimulating effect of NH₄⁺ on heterotrophic CO₂ fixation permitted experimental control of the fixation. Further information concerning the pathways of dark CO₂ fixation might be gained by studying the specific products of such NH₄⁺-controlled fixation.

MATERIALS AND METHODS

*E. gracilis* was grown as described previously (12) in a mineral medium modified from the low pH phototrophic medium of Hutner *et al.* (6). The carbon source for phototrophic growth was 5% CO₂ in air, perfused slowly through the culture at a constant rate. The cells were grown at 25 C in a constant environment room with continuous overhead illumination of intensity 2,000 ft-c at the position of the flasks. The carbon source for organotrophic growth was 1% glucose, and these cells were grown at 25 C in complete darkness. To investigate the products of dark CO₂ fixation, *Euglena* were grown to appropriate cell densities (3.0 × 10⁶ cells/ml for phototrophic cells, 5.0 × 10⁶ cells/ml for organotrophic cells). Half of the cells were then subjected to NH₄⁺ stimulation, as described below, and the other half comprised the controls. Cells (100 ml) were harvested, washed, and resuspended in 10 ml of the appropriate medium. Each cell suspension was pipetted into a brown, foil-wrapped flask which was then sealed with a serum stopper. Five μCi NaH¹⁴CO₃ (54.9 mCi/mmol) were injected into each flask with a Hamilton syringe and the flasks shaken at 25 C. The low pH of the medium (pH 4.7) ensured that all ¹⁴CO₂ was liberated. After 10 min, the entire content of each flask was poured into 40 ml boiling absolute ethanol. The disrupted cells were filtered and the filtrate was evaporated to dryness and redissolved in 2 ml 80% ethanol.

Aliquots (50 μl) were applied to squares of Whatman No. 1 chromatography paper (20 × 20 cm) and subjected to two-dimensional ascending chromatography using the solvent systems of Benson *et al.* (1). Radioactive areas were located autoradiographically by use of x-ray film. Individual compounds were tentatively identified by comparison with standards, and then eluted and subjected to co-chromatography with known standards for final identification.

Aliquots of the eluates were counted in a Beckman liquid scintillation counter to quantify the label incorporated into each compound. For NH₄⁺ stimulation, cells were grown to the appropriate cell density, harvested, and resuspended for 24 h in medium lacking NH₄⁺ (NH₄⁺ starvation), then resuspended in 1 mM KH₂PO₄ containing 10 mM NH₄Cl (NH₄⁺ replenishment) for dark CO₂ fixation. This is the experimental protocol (13) for maximal stimulation of the heterotrophic CO₂ fixation by NH₄⁺. In the case of control cells, CO₂ fixation was carried out in 1 mM KH₂PO₄ (pH 4.7). Na₂¹⁴CO₃ was obtained from the Radiochemical Centre, Amersham, England. Scintillation chemicals were obtained from Beckman. All other chemicals were supplied by Sigma or British Drug Houses.

RESULTS

Figure 1 is a tracing of an autoradiograph of a typical chromatogram which shows the separation of the radioactive products of NH₄⁺-stimulated dark CO₂ fixation after phototrophic growth.
amino acids, glutamine, glycine, alanine, serine, and the unidentified nucleotides. Smaller increases were observed after NH₄⁺ treatment for all of the other compounds with the exception of PEP. Some compounds, which were not detected in the control cells, were detected after NH₄⁺ treatment, for example malate, succinate, and serine. In both the control and NH₄⁺-stimulated cells, glutamate was a major product of dark CO₂ fixation. NH₄⁺ treatment stimulated incorporation of CO₂ into glutamate by only 25%, compared with 97% stimulation of glycine production.

**DISCUSSION**

It has been suggested previously (13) that the regulation of heterotrophic CO₂ fixation by NH₄⁺ supports the possibility that a major function of the fixation is in anaerobic replenishment of the tricarboxylic acid cycle. After NH₄⁺ starvation, exposure to further supplies of NH₄⁺ could result in an increase in amino acid biosynthesis by amination of, for example, α-keto acids, draining them from the tricarboxylic acid cycle. Heterotrophic carboxylation could then be stimulated to replenish these acids. In this case it would be expected that the extra carbon fixed after NH₄⁺ stimulation would be found in amino acids derived from tricarboxylic acid cycle intermediates.

As shown in Table I, NH₄⁺ treatment resulted in an overall increase in the amount of ¹⁴C labelled CO₂, and a high proportion of the extra counts was found in glutamine, with smaller increases in incorporation of labeled CO₂ into glutamate and aspartate. These results are consistent with the fact that glutamine, in both plants and animals, acts as a nitrogen store in times when NH₄⁺ is plentiful. The labeled CO₂ must have entered the amino acids via the tricarboxylic acid cycle, since α-ketoglutarate is the precursor for the synthesis of glutamate and glutamine, and OAA provides the skeleton for the synthesis of aspartate. Moreover, labeled CO₂ was found in the tricarboxylic acid cycle intermediates, malate, citrate, and succinate. These results are similar to those obtained by Miyachi et al. (9) in Chlorella. They found that NH₄⁺ stimulation altered the spectrum of products of dark CO₂ fixation, causing in particular, increases in incorporation of radioactivity into glutamate and glutamine. Aspartate was found to be a major product in both control and stimulated cells.

The most likely route by which CO₂ enters the tricarboxylic cycle is via the synthesis of aspartate, followed by the incorporation of CO₂ into glutamine, and then into the tricarboxylic acid cycle.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Glycine</td>
<td>467</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1,720</td>
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<tr>
<td>Glutamate</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Serine</td>
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<tr>
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</tr>
<tr>
<td>Citrulline</td>
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</tr>
<tr>
<td>Citrate</td>
<td>133</td>
</tr>
<tr>
<td>Malate</td>
<td>0</td>
</tr>
<tr>
<td>Succinate</td>
<td>0</td>
</tr>
<tr>
<td>Sugar phosphates</td>
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</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>1,840</td>
</tr>
<tr>
<td>Unidentified nucleotides</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>20,450</td>
</tr>
</tbody>
</table>

Abbreviations: PGA: 3-phosphoglycerate; PEP: phosphoenolpyruvate; OAA: oxaloacetic acid.
acid cycle is by way of the carboxylation of PEP, catalyzed by PEP carboxylase (EC 4.1.1.31), an enzyme which is known to be active in Euglena (11).

It was also shown that NH₄⁺ stimulation caused increased incorporation of labeled CO₂ into other amino acids not derived from tricarboxylic acid cycle intermediates, for example glycine, serine, and alanine, which are all derived from the glycolytic pathway. This was not observed by Miyachi et al. (9) in Chlorella. The largest increase in incorporation of labeled CO₂ after NH₄⁺ treatment was into glycine. Glycine and serine, which are readily interconvertible, are synthesized from PGA which was also observed to be a product of dark CO₂ fixation. (The amino groups are used for the formation of these amino acids, as well as glutamine, are derived from glutamate. This may be why glutamate itself did not increase after NH₄⁺ treatment, since extra glutamate was utilized in formation of glutamine, glycine, etc., and did not accumulate.) These findings tend to suggest the support of Lynch and Calvin (8) that Euglena fixes CO₂ heterotrophically by more than one route. The pathway by which CO₂ first enters PGA is still not certain. Moses et al. (10) proposed for Zygorycton chlorella that this might be by reversal of the decarboxylation of 6-P-glucuronate to a pentose phosphate. Although incorporation of labeled CO₂ into 6-P-glucuronate so far has not been shown in Euglena, the enzyme catalyzing the reaction, P-glucuronate dehydrogenase (EC 1.1.1.44), has been shown to be present (15).

In organisms which use the reactions of the glyoxylate shunt, the main precursor of glycine (and serine) may be glyoxyxlate. Euglena is known to possess the glyoxylate shunt enzymes, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), when grown organotrophically on acetate or ethanol (2, 3) and to a lesser extent when glucose is the carbon source (4, 14). It is feasible that under these conditions CO₂ could enter glycine and serine not by way of PGA, but first via the tricarboxylic acid cycle intermediates OAA and isocitrate which are glyoxylate precursors. In this case it would not be necessary to postulate a second route of entry of CO₂, since presumably CO₂ would enter the tricarboxylic acid cycle in the same way as was suggested above. However, there are some serious objections to this pathway of CO₂ incorporation in the case of Euglena. Although the glyoxylate cycle enzymes are active in Euglena cultured organotrophically on acetate (and to a lesser extent on glucose) these enzymes were not detected in Euglena grown phototrophically (4, 14). (Malate synthase may be induced in phototrophic cultures by the addition of acetate but Cook and Carver [2] found that this induction is partially inhibited by the continued presence of visible light. This finding was not confirmed, however, by Woodward and Merrett [16].) Since glycine was found to be a major product of organotrophic CO₂ fixation after NH₄⁺ stimulation in both phototrophically and organotrophically grown Euglena, it seems more likely that the pathway of glycine formation is by way of PGA. Although the exact pathway for the incorporation of CO₂ is still not known, our results indicate that heterotrophic CO₂ fixation, as well as possibly functioning in anaerobic replenishment, is also involved in the building of carbon skeletons for the formation of amino acids such as glycine and serine, and that this component of the fixation is also regulated by NH₄⁺.

A small percentage of labeled CO₂ was fixed into citrulline in the NH₄⁺-stimulated cells, suggesting that the availability of NH₄⁺ may also have stimulated reactions of the urea cycle which include the carboxylation of arginine to citrulline. This explanation was offered by Hiller previously (5) to explain his observed stimulation of CO₂ fixation into sitrulline and arginine in Chlorella; however, ethionine of this pathway have not as yet been demonstrated for Euglena.

By use of the knowledge that NH₄⁺ stimulates heterotrophic CO₂ fixation in Euglena, it has been possible to show that CO₂ fixed heterotrophically by this organism is mainly involved in the formation of carbon skeletons for amino acid synthesis, both by way of the tricarboxylic acid cycle and by other, less well defined routes.

LITERATURE CITED