Effects of Glucose Feeding on Respiration and Photosynthesis in Photoautotrophic Dianthus caryophyllus Cells

Mass Spectrometric Determination of Gas Exchange

Marie-Hélène Avelange, Frédéric Sarrey, and Fabrice Rébillé*
Département de Biologie, Service de Radioagronomie, C.E.N. Cadarache, 13108 St. Paul Lez Durance, Cedex, France

ABSTRACT

When glucose (20 millimolar) was added to photoautotrophic cell suspension cultures of Dianthus caryophyllus, there was during the first 10 hours an accumulation of carbohydrates and phosphorylated compounds. These biochemical changes were accompanied by a progressive decrease of net photosynthesis and a twofold increase of the dark respiratory rate. The rise of respiration was associated with a rise of fumarase and cytochrome c oxidase activities, two mitochondrial markers. Gas exchange of illuminated cells were performed with a mass spectrometry technique and clearly established that during the first hours of glucose feeding, the decrease of net photosynthesis was essentially due to an increase of respiration in light, whereas the photosynthetic processes (gross O₂ evolution and gross CO₂ fixation) were almost not affected. However, after 24 hours of experiment, O₂ evolution and CO₂ fixation started to decline in turn. While ribulose-1,5-bisphosphate carboxylase activity was little affected during the first 48 hours of the experiment, the maximal light-induced phosphoribulokinase activity dramatically decreased with time and represented after 48 hours only 30% of its initial activity. It is postulated that the decrease in phosphoribulokinase activity was at least partially responsible for the decrease of CO₂ fixation and the metabolic events involved in this regulation are discussed.

Among the numerous factors that modulate the photosynthetic activity (such as light, temperature, CO₂, and O₂ concentrations), the partitioning and utilization of the photosynthetic within the plant (i.e. the interaction of 'source' and 'sink') is often regarded as an overriding factor. Indeed, the capacity for photosynthetic carbon assimilation is related to the capacity of cytoplasm to synthesize and export (or store) sucrose and to the capacity of the stroma to produce starch (10). In this context, it was reported that addition of sucrose in the external medium of chlorophyllous higher plant cell cultures inhibited photosynthesis and chloroplast development (6, 8, 21) and that maximal rates of photosynthesis were correlated with low cellular sucrose/glucose content (24).

Direct feedback inhibition of sucrose on the enzymes of sucrose synthesis (12) is generally accepted to be a main facet of this interaction, with Pi as a central regulatory metabolite (29). In addition, the fact that glucose, and to a lesser extent fructose, has similar effects to sucrose on photosynthesis (27) lead to the hypothesis of an 'overflow' loop around the pathway of sucrose synthesis, involving an increase of hexosesphosphate, a stimulated synthesis of fructose 2,6-P, and a decrease of cytosolic Pi (9).

However, a high intracellular carbohydrate level was often correlated with a high mitochondrial activity (4, 26), and it was reported that addition of sugars in the external medium of chlorophyllous cell suspension cultures increased the respiratory rate (24). Thus, a stimulated O₂ consumption due to a high carbohydrate level might contribute to an apparent decrease of photosynthesis when it is measured as net O₂ exchange.

In the present paper, we report a study of the effects of glucose feeding on photosynthesis and respiration of photoautotrophic suspension culture of carnation cells. The different gas exchange processes occurring in the light were discriminated using ¹⁸O and ¹³C isotopes and a mass spectrometry technique.

MATERIALS AND METHODS

Plant Material

Photoautotrophic cell suspension cultures of carnation (Dianthus caryophyllus L.) were routinely subcultured every 2 weeks using the Murashige and Skoog medium supplemented with growth regulators, as previously described (24). The cell suspension (200 mL) was bubbled with air (5 L·h⁻¹) either in 500 mL conical flasks agitated on an orbital shaker at 100 rpm or in a 1 L bubble-column. Flasks and columns were illuminated continuously with fluorescent lamps at a light intensity of 150 to 180 µE·m⁻²·s⁻¹ (400–700 nm) and room temperature was kept constant at 25°C. Under these conditions the photoautotrophic cell suspensions appeared as small aggregates of 100 to 150 cells, and the cell doubling time was approximately 15 to 20 d. Cells were harvested in the midlog exponential phase of growth for the experiments. All the experiments were reproduced at least three times. Respiratory and photosynthetic activities varied depending
on the sample. Typical rates were in the range 0.08 to 0.20 nmol min⁻¹ mg⁻¹ fresh weight for respiration and 0.22 to 0.34 nmol min⁻¹ mg⁻¹ fresh weight for net photosynthesis. The Chl content varied in the range 0.1 to 0.15 μg mg⁻¹ fresh weight.

**Chl Determination**

Cells (300 mg fresh weight) were removed, strained on a fiberglass filter, and dropped in 10 mL of 80% acetone. After vigorous homogenization the Chl concentration was determined according to Arnon (3).

**Gas Exchange Measurements**

Respiration and net photosynthetic activities were determined polarographically using a Clark-type electrode (Hansatech, Norfolk, V.K.). Light (800 μE m⁻² s⁻¹) was supplied with a projector equipped with a 150 W halogen lamp. For analysis of unidirectional O₂ and CO₂ fluxes, the cell suspension (1.5 mL) was transferred into a thermostated (25°C) reaction vessel. A Teflon membrane at the bottom of the reaction vessel allowed the dissolved gases to be directly introduced into a three-collector mass spectrometer (V.G. Instruments, MM 14-80). Light conditions were the same as described above. For O₂ and CO₂ exchange determinations, 2 mL of ¹⁵O₂ and 0.15 mL of ¹³CO₂ (99% ¹⁸O and 99% ¹³C, purchased from C.E.A. Saclay, France) were bubbled in the cell suspension before to close the reaction vessel. In these conditions the initial O₂ and CO₂ concentrations were, respectively, 350 and 250 μM. This high CO₂ level was saturating for photosynthesis and strongly reduced the operation of the photorespiratory pathway. Oxygen and carbon dioxide uptake and evolution were measured by recording simultaneously m/e 32 and m/e 36 with the first and second collectors respectively, then after peak jumping m/e 44 and m/e 45 with the second and third collectors, respectively. The time of integration was 1 s. Calculations were made according to the equation of Radmer and Kok (23).

**Biochemical Analysis**

Cells (200–500 mg fresh weight) were strained on a glass-fiber filter and rinsed with distilled water. Alkaline and acidic fixation were used, respectively, for carbohydrates and phosphorylated sugars determinations. For carbohydrate determinations, cells were dropped into 3 mL of ice-cold 0.5 N NaOH and left 15 min in ice to allow a complete fixation. Samples were centrifuged for 10 min at 2000g and the supernatants were neutralized with HCl. In neutralized supernatants, glucose, fructose, and sucrose were assayed spectrometrically as previously described (24). For starch analysis, 1 mL of the alkaline extract was stirred in a Potter-Elvehjem homogenizer, then neutralized. A sample was incubated for 1 h at 35°C with an equal volume of amylglucosidase (1 mg mL⁻¹) in sodium acetate (pH 4.6). Glucose from starch hydrolysis was determined as above. Fructose was also measured to appreciate potential sucrose cleavage. For phosphorylated sugar determinations, cells were dropped into 3 mL of ice cold perchloric acid (5%). Samples were centrifuged for 10 min at 2000g and the supernatants were neutralized with KOH. G₆P, F₆P, 3-PGA were determined according to Czok (7) and Lane and Michal (18).

Intracellular Pi determinations were undertaken as previously described (25).

For measurement of enzymic activities, all assays were first performed on blanks to detect any nonspecific drift or endogenous substrate or enzyme contamination. We verified that the activities were linear with respect to time for at least 2 min and were proportional to the amount of broken cells. For fumarase and Cyt c oxidase activities, cells (about 600 mg fresh weight) were disrupted in 3 mL of a ice-cold buffer containing 0.1 mM Pi, 0.05% Triton X-100 (pH 7.4), rapidly centrifuged 5 min at 2000g, and the supernatant was assayed in presence of 50 mM malate (fumarase) (5) or 50 μM reduced

---

1 Abbreviations: F₆P, fructose 6-phosphate; G₆P, glucose 6-phosphate; 3-PGA, 3-phosphoglycerate; CCP, carbonyl m-chlorophenylhydrazone; PRK, phosphoribulokinase; RuBPCase, ribulose-1,5-bisphosphate carboxylase.

---

**Figure 1.** Time course changes in the level of intracellular carbohydrates and phosphorylated compounds after supplying 20 mM glucose to a photoautotrophic suspension cell of D. carophyllus. A, Glucose, fructose, sucrose; B, G₆P, F₆P, 3-PGA, and starch.
Cyt c (30). For RuBPCase and PRK activities, 10 mL of the cell suspensions (about 800 mg fresh weight) were stirred 5 min under illumination (500 μE·m⁻²·s⁻¹). After a quick straining on a fiberglass filter, the cells were rapidly dropped in liquid N₂, then disrupted in 2 mL of the following ice-cold grinding medium: 150 mm Tris, HCl (pH 7.8), 5 mm DTT, 1.5% polyclar AT, 1 mm PMSF, 5 mm MgCl₂, and 0.1 mm EDTA (RuBPCase) or 10 mm MgSO₄ and 50 mm KCl (PRK). After 1 min centrifugation in a Beckman minifuge the supernatant was immediately assayed. The PRK activity was assayed spectrophotometrically in the grinding medium without DTT according to Kagawa (16). The RuBPCase activity was measured radiometrically according to Lorimer (19). Of the supernatant 50 μL was incubated in presence of 250 μL of 0.2 m Bicine (pH 8.2), 10 mm DTE, 2 mm EDTA, 33 mm MgCl₂, 50 μL RuBP 50 mm, 50 μL NaH¹⁴CO₃ 70 mm (2.5 μCi/mol). The reaction was allowed to proceed at room temperature and terminated at 15, 30, 45, and 60 s by addition of 200 μL 2 N HCl. Samples were dried overnight at 60°C, and radioactivity was determined with a liquid scintillation spectrometer. The activity was calculated from the linear part of the curve. The full activity of the RuBPCase was measured in a same way after 10 min incubation at room temperature in presence of 10 mm NaHCO₃, 20 mm MgCl₂.

RESULTS

Carbohydrate Contents and Net O₂ Exchange

— When 20 mm glucose were added in the culture medium, glucose was incorporated in the cells without a lag phase and at a rate of approximately 75 nmol min⁻¹·mg⁻¹ Chl (result not shown). In our experimental conditions, the glucose concentration in the external medium remained high (above 15 mm) throughout the experiment. During the first 10 h, there was a marked increase of the intracellular glucose, fructose and sucrose content (Fig. 1A). Likewise, the maximal content of phosphorylated compounds such as G₃P, F₆P, and 3-PGA was reached after 8 to 12 h of glucose feeding (Fig. 1B). In contrast, starch accumulation required a longer time (Fig. 1A). These biochemical changes were accompanied by a decrease of net photosynthesis and a progressive increase of the respiratory rate recorded before illumination (Fig. 2). Similar results were also obtained when 20 mm sucrose, instead of glucose, were added in the culture medium (results not shown). Similar observations resulting from sucrose feeding have also been previously reported with CO₂-grown carnation cells (24).

As shown in Table I, the basal rate of respiration in dark was always lower (2–3 times lower) than the uncoupled rate in the first 48 h following glucose addition. This is indicative of a respiratory control which limited electron flow and glycolysis in response to a high ATP/ADP ratio (2, 13, 26). The higher uncoupled respiratory rate recorded after glucose addition could be attributed to an increased availability of respiratory substrates. Alternatively, it is possible that the rise of respiratory rates was, at least partially, correlated with a higher amount of respiratory enzymes. From this point of view we observed that maximal fumarase and Cyt c oxidase activities, two mitochondrial markers, increased more than two times after 48 h of glucose feeding (Table I), a result compatible with this hypothesis.

The decrease of net photosynthesis was not ascribable to a variation of the Chl content which remained constant during the time course of the experiment. Therefore it could be the result of either the stimulation of mitochondrial respiration in the light or feedback inhibition through metabolic changes and ‘source-sink’ interactions (9). To test the first proposition, we measured simultaneously O₂ and CO₂ gas exchange under such conditions.

O₂ and CO₂ Gas Exchange in the Light

O₂ and CO₂ gas exchange was undertaken in presence of a saturating level of CO₂ for photosynthesis. In light the rate of CO₂ uptake represented approximately 75% of the rate of O₂.

Table I. Effect of Glucose Addition upon Respiratory Rates, Fumarase, and Cyt c Oxidase Activities

These values are averages of three different sets of experiments.

<table>
<thead>
<tr>
<th>Time after Glucose Addition</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol min⁻¹·mg⁻¹ Chl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O₂ uptake rate</td>
<td>1 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Uncoupled O₂ uptake rate (+</td>
<td>2.5 ± 0.3</td>
<td>4.2 ± 0.4</td>
<td>5 ± 0.6</td>
</tr>
<tr>
<td>CCCP 2 μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumarase</td>
<td>3 ± 0.15</td>
<td>5 ± 0.5</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>Cyt c oxidase</td>
<td>0.50 ± 0.10</td>
<td>0.75 ± 0.10</td>
<td>1.10 ± 0.25</td>
</tr>
</tbody>
</table>
evolution, indicating that at least 75% of the reducing power produced by the photosynthetic apparatus was diverted toward CO₂ fixation (Fig. 3). This is a minimal value depending of the amount of respiratory CO₂ recycled within the cell. During a dark-to-light transition, the O₂ uptake remained almost unchanged, whereas the CO₂ efflux was strongly inhibited. It is not clear at the present stage whether this inhibition was representative of a decrease of substrate decarboxylations or the result of CO₂ recycling. This point is currently under investigation.

As shown in Figure 4, addition of glucose in the external medium resulted in a similar increase of both CO₂ release and O₂ uptake in the light. Such a result was indicative of a stimulation of the mitochondrial respiratory activity. The maximal O₂ uptake and CO₂ efflux were reached after 20 to 24 h of experiment and slightly decline thereafter (Fig. 4). During these first 20 to 24 h of glucose feeding, there was no significant decrease of the O₂ evolution and CO₂ uptake rates (Fig. 5). After this time, however, both rates started to decline. As expected, net O₂ evolution and net CO₂ uptake were tightly coupled, and the two curves remained superimposed throughout the experiment (Fig. 5).

Measurements of the activities in light of RuBPCase and PRK, two marker enzymes of the Calvin cycle activity, are presented in Table II. The average of four different sets of experiment indicates that the RuBPCase activity remained almost unchanged during the first 48 h following glucose addition. In one case, however, we observed between 24 and 48 h a slight decrease (about 30%) of the full activity (i.e. the activity measured after incubation in presence of NaHCO₃ and Mg²⁺), but not of the light-induced activity which is presumably representative of the in vivo status. These results indicate that RuBPCase activity did not change significantly during the first 2 d following glucose addition. However, this activity might decrease after longer times since it was reported that heterotrophic cells have a lower RuBPCase content than autotrophic cells (20). In contrast with the RuBPCase, the maximal light-induced PRK activity decreased with time and represented after 48 h only 30% of the initial activity (Table II). Because Pi deficiency was proposed to be a main facet of source-sink interaction, the intracellular Pi level was measured in the same conditions. As shown in Table II, the Pi pool was not lowered by glucose feeding but slightly increased with time.

**DISCUSSION**

Our results indicate that addition of glucose in the culture medium of actively dividing photoautotrophic carnation cells resulted in a rapid increase of soluble carbohydrates and glycolytic products such as G₆P, F₆P, and 3-PGA, a rise of the dark respiration rate and a decrease of net photosynthesis.
The effect of glucose supply on the fumarase and Cyt c oxidase activities suggests that the stimulation of the respiratory rate was correlated with either an increase of the amount of respiratory enzymes per mitochondria or an increase of the number of mitochondria per cell. These two assumptions are also supported by the fact that uncoupled and basal respiratory rates remained roughly in the same ratio during the experiment. Previous studies (14) have shown that neither the overall capacity of the glycolytic enzymes nor the availability of substrates for Krebs cycle were ultimately responsible for determining the rate of uncoupled respiration in sycamore cells. In these cells the decrease of the uncoupled respiration during sucrose deprivation was attributable to a decrease of the mitochondrial number rather than the availability of substrates (15), and this was fully reversible by addition of sucrose. Therefore, it is likely that, for carnation cells in midlog exponential phase of growth, the number of mitochondria per cell was correlated with the availability of respiratory substrates. From this point of view it must be noted that CO₂-grown carnation cells had a higher carbohydrate content but also a higher fumarase activity than air-grown cells (24).

![Graph](image)

**Figure 5.** Effects of glucose feeding on O₂ evolution and CO₂ uptake rates in the light after glucose supply. The experimental procedure was as described in Figure 3. Eo₂, O₂ evolution; UCO₂, CO₂ uptake; NO₂, net O₂ exchange; NCO₂, net CO₂ exchange.

### Table II. Effect of Glucose Addition upon the RuBPCase and PRK Activities

The light activation for both enzymes was undertaken by illumination of the cell suspension for 5 min at 500 µE · m⁻² · s⁻¹. These values are an average of four different sets of experiments.

<table>
<thead>
<tr>
<th>Time after Glucose Addition</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>RuBPCase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light activated</td>
<td>2.10 ± 0.33</td>
<td>2.50 ± 0.39</td>
<td>1.90 ± 0.10</td>
</tr>
<tr>
<td>Fully activated</td>
<td>4.10 ± 1</td>
<td>4.40 ± 0.75</td>
<td>4 ± 1.50</td>
</tr>
<tr>
<td>PRK</td>
<td>22.5 ± 3.8</td>
<td>14.1 ± 2.3</td>
<td>6.7 ± 1.1</td>
</tr>
</tbody>
</table>

Mass spectrometric measurements clearly established that, during the first hours of glucose feeding, the decrease of net photosynthesis (net O₂ evolution and net CO₂ uptake) was essentially due to an increase of respiration in light, whereas the photosynthetic processes remained unchanged. After 20 h, the rate of CO₂ fixation which was presumably representative of the Calvin cycle activity, started to decline together with the rate of gross photosynthesis, which measured the rate of electron transfer in the chloroplastic chain.

It was suggested that the inhibition of the Calvin cycle activity following glucose or sucrose addition could be ascribable to a decrease of the cytosolic Pi concentration (9). This hypothesis relies on the fact that an increasing level of phosphorylated products would ultimately lower the cytosolic Pi pool as mimicked with Pi sequestering agents such as mannose (28). In this situation the essential recycling of Pi would be prohibited leading to starch accumulation and an inhibition of photosynthesis (29). In our experimental conditions, however, 1 mM Pi was present in the culture medium. Taking into account the high performances of the Pi translocator located on the plasmalema of higher plant cells (25), it is doubtful that a cytosolic Pi depletion could alone explain the CO₂ uptake inhibition. In addition, no decrease of the total Pi pool could be detected in presence of glucose. On the other hand, our results indicate that the decline of the Calvin cycle was associated with a decrease of the light-induced PRK activity. This enzyme is light-activated via thioredoxin (22) and is known to be regulated by stromal metabolite levels (11). In particular, 6-phosphogluconate (1, 11) and 3-phosphoglycerate (17) are potent inhibitors of this enzyme. The molecular basis of the regulatory processes involved in the feedback modulation of photosynthesis, described here, remain to be clearly determined. However, it may be postulated that a high 3-phosphoglycerate level in the chloroplasts resulted in a decrease in PRK activity and in a limitation in the availability of reducing power in the stroma.

### ACKNOWLEDGMENTS

We thank Drs. A. Vermeglio and P. Thibault for helpful discussions.

### LITERATURE CITED