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# Adenylate ratios in the cytosol, chloroplasts and mitochondria of barley leaf protoplasts during photosynthesis at different carbon dioxide concentrations

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Barley (*Hordeum vulgare*) protoplasts were incubated in darkness and in the light at saturating and limiting $CO_2$ concentrations. The protoplasts were fractioned by a membrane filtration technique which allows quenching of the metabolism by acidification within about 0.1 s and the ATP/ADP ratios in the cytasol, chloroplasts and mitochondria were determined. It is concluded that the cytosolic ATP/ADP ratio is considerably higher during photosynthesis at limiting $CO_2$ (which is the normal situation for a $C_3$ plant in air) compared to photosynthesis at saturating $CO_2$ or darkness.

ATP/ADP ratio; Chloroplast; Cytosol; Mitochondria; Protoplast

## 1. INTRODUCTION

Adenylates participate in, and regulate, many reactions in the cell and are therefore very important metabolites. The net transport of adenylates between chloroplasts, mitochondria and cytosol is slow and therefore the adenylates form more or less separate pools in these cell compartments [1]. These pools do, however, communicate, most importantly via the mitochondrial adenylate translocator [2] and the 3-phosphoglycerate-triosephosphate shuttle in the chloroplast envelope [3]. Knowledge about the phosphorylation level of the adenylates in the different cell compartments can thus give important information about the metabolic state of the cell.

In this study the technique of rapid fractionation of protoplasts by membrane filtration developed by Lilley et al. [4] was used to study the effect of light at saturating and limiting $CO_2$ concentrations on the ATP/ADP ratios in the cytosol, mitochondria and chloroplasts of barley protoplasts.

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*Abbreviations:* NADP-TPD, NADP-triosephosphate dehydrogenase; PEP, phospho*enol*pyruvate

## 2. MATERIALS AND METHODS

Protoplasts were isolated from 6- to 10-day-old barley leaves (*Hordeum vulgare* var. Gunilla). Leaves were cut into small segments and digested for 2 h in digestion medium: 1% cellulase (Cellulysin from Calbiochem), 0.3% pectinase (Macerase from Calbiochem), 0.2% BSA, 0.05% PVP, 0.5 M sorbitol, pH 5.0, at 28°C. Intact protoplasts were collected by sedimentation followed by flotation essentially as described by Edwards et al. [5].

Experiments were conducted in an assay medium containing: 0.25 M sucrose, 0.25 M sorbitol, 10 mM KCl, 0.5 mM $MgCl_2$, 0.06% BSA, 0.2% PVP, 10 mM Hepes (pH 7.2). The assay medium is similar to the medium used by Stitt et al. [6] and has the same density as the protoplasts.

Fractionation experiments were performed using specially made equipment basically according to



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Table 1

The filter combinations used to obtain the three different filtrates

| Filter | Filtrate | | |
|---|---|---|---|
| | F0 Total extract | F1 Total extract – chloropl. | F2 Total extract – chloropl. – mito. |
| 15 μm nylon net | X | X | X |
| 8 μm membrane filter | | X | X |
| 0.45 μm membrane filter | | | X |

Lilley et al. [4]. Protoplasts (20 μg Chl/ml) were incubated in three 5 ml glass syringes fitted to filter holders with different filter combinations giving three different filtrates according to table 1. Protoplast rupture was obtained using a 15 μm nylon net (Henry Simon Ltd., Stockport, England). Chloroplasts and mitochondria were collected on 8 and 0.45 μm membrane filters, respectively (Millipore MF). Each filtrate was divided into three parts and buffer or trichloroacetic acid was injected into the filtrates from 1 ml plastic syringes closed at the same time as the protoplast syringes making the fluids mix instantaneously. The native filtrate was used for the measurement of marker enzymes and in the acidic filtrate ATP and ADP were determined. The flow rate was 3.6 ml/s and the volume inside the filter holders was about 400 μl. Thus the sample was mixed with acid within about 0.1 s.

Marker enzymes used were: fumarase for mitochondria [7], PEP carboxylase for cytosol [8] and NADP-TPD for chloroplasts [9]; chlorophyll was measured according to Bruinsma [10]. ATP was assayed by the firefly luciferase method.

## 3. RESULTS

The recovery of marker enzymes in the filtrates is shown in table 2. The 8 μm filter is very efficient in removing chloroplasts as close to 90% of the chloroplast marker enzyme NADP-TPD is lost on this filter. Also on the 8 μm filter some mitochondria (26%) are lost. The 0.45 μm filter removes almost all of the remaining mitochondria. The high retention of the marker enzymes for chloroplasts and mitochondria on the filters indicates a very low organelle breakage in the filtration.

The ATP/ADP ratios in the different cell compartments in darkness and in light at saturating $CO_2$ are shown in table 3. The ratio between ATP and ADP in the total extract (F0) is somewhat higher in the light than in the dark. The increase in ATP is confined to chloroplasts and mitochondria whereas the ATP/ADP ratio in the cytosol is similar or even lower in the light compared to the dark. This is in agreement with the results obtained by Stitt et al. [6] for wheat protoplasts under similar conditions.

An experimental situation giving a limiting $CO_2$ supply to protoplasts was obtained by continuous addition of a bicarbonate solution to a $CO_2$-free assay medium. By using this system a constant rate

Table 2

The distribution of marker enzymes in the different filtrates

| Filtrate | Fumarase | PEPcase | NADP-TPD |
|---|---|---|---|
| F0 | 100 | 100 | 100 |
| F1 | 74 ± 7 | 91 ± 7 | 12 ± 3 |
| F2 | 7 ± 2 | 81 ± 7 | 9 ± 3 |

The activities in F1 and F2 are expressed as % of the activity in F0. The specific activity in the total extract (F0) was: fumarase 27 ± 4, PEP carboxylase 36 ± 8 and NADP-TPD 589 ± 164 μmol/mg Chl per h. The values are the mean from 30 fractionation experiments

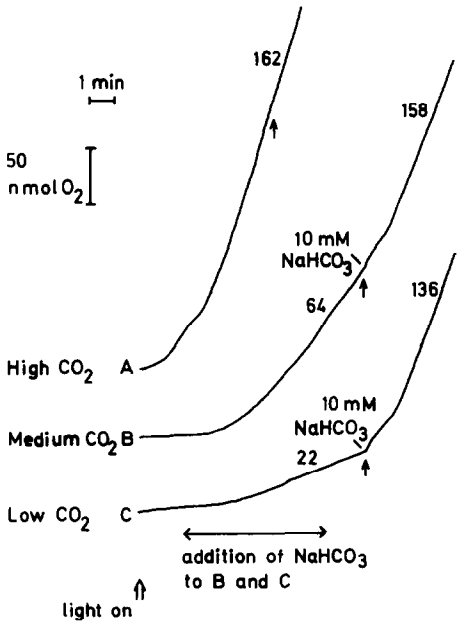
Table 3

The ATP/ADP ratios in chloroplasts, mitochondria and cytosol in the dark, in the light at saturating $CO_2$ (10 mM $NaHCO_3$) and in the light at limiting $CO_2$ (according to fig.1)

| | ATP/ADP | | | |
|---|---|---|---|---|
| | Total extract (F0) | Chloroplasts (F0 – F1) | Mitochondria (F1 – F2) | Cytosol (F2) |
| Dark (9) | 1.0 ± 0.3 | 0.6 ± 0.2 | 0.8 ± 0.3 | 3.0 ± 1.0 |
| Light | | | | |
| High $CO_2$ (10) | 1.4 ± 0.6 | 1.1 ± 0.4 | 1.5 ± 0.6 | 2.8 ± 0.4 |
| Medium $CO_2$ (5) | 3.7 ± 0.7 | 3.2 ± 0.8 | 2.6 ± 1.1 | 7.2 ± 1.6 |
| Low $CO_2$ (5) | 4.0 ± 0.5 | 3.1 ± 0.5 | 4.2 ± 1.1 | 7.5 ± 1.7 |

The rate of photosynthetic oxygen production at limiting $CO_2$ compared to high $CO_2$ was 40–50% for 'medium $CO_2$', and 15–20% for 'low $CO_2$'. Saturating light (500 $\mu E \cdot m^{-2} \cdot s^{-1}$) was used. In parentheses are the number of experiments

of photosynthetic $O_2$ evolution was obtained after illumination for a couple of minutes (fig.1). This experimental approach was used in experiments where the protoplasts were fractionated during steady-state photosynthesis at limiting $CO_2$ (table 3).




The ATP/ADP ratio in the total extract increased as $CO_2$ became limiting for the photosynthetic rate. An increased ATP/ADP ratio was observed in all cell compartments at limiting $CO_2$ compared to saturating $CO_2$. Of special interest is the very pronounced increase obtained in the ATP/ADP ratio in the cytosol as the $CO_2$ concentration became limiting. Thus, the cytosolic ATP/ADP ratio is higher in the light at limiting $CO_2$ compared to that in darkness (table 3). This is in contrast to the suggestion by Hampp et al. [11] that the cytosolic energy state is kept at a constant, preset value by collaboration between photosynthetic and oxidative phosphorylation. In darkness the same ATP/ADP ratio was obtained

Fig.1. Photosynthesis of barley protoplasts at different $CO_2$ concentrations. (A) 10 mM $NaHCO_3$ was added before the light was turned on (high $CO_2$). (B) After 2 min in the light $NaHCO_3$ was added for 5 min at a rate of 135 $\mu$mol/mg Chl per h (medium $CO_2$). (C) After 2 min in the light $NaHCO_3$ was added for 5 min at a rate of 45 $\mu$mol/mg Chl per h (low $CO_2$). For B and C 10 mM $NaHCO_3$ was added to give saturating $CO_2$ 1 min after the addition of limiting $CO_2$ had stopped. Numbers on the traces refer to $\mu$mol $O_2$/mg Chl per h. The arrow (↑) refers to time of fractionation in parallel experiments (see table 3).

both at high $CO_2$ and low $CO_2$ concentrations (not shown). The changes of the ATP/ADP ratio in the chloroplasts as a consequence of different $CO_2$ concentrations agree well with results reported by Dietz and Heber [12] using a non-aqueous method to obtain ATP/ADP ratios in spinach chloroplasts.

## 4. DISCUSSION

The turnover time for adenylates has been estimated to be of the order of 1 s or less [1]. Thus conventional methods for cell fractionation cannot be used for the determination of ATP/ADP ratios in different cell compartments. The application of membrane filtration for fractionation of protoplasts is at present the quickest method available to estimate intracellular metabolite concentrations [4]. As the metabolism is stopped by acidification within about 0.1 s the method should allow determination of metabolites which have a rapid turnover.

In the present study the metabolite levels in the different filtrates were used without correction for cross-contamination. This was made possible by the high efficiency of the filtration (table 2). This method will not give true absolute values for the ATP/ADP ratios but should nevertheless allow meaningful comparisons between different treatments to be made. Since the values for chloroplasts and mitochondria are calculated from the difference between filtrates only differences in adsorption of marker enzymes, difference in organelle breakage and so on will contribute to the experimental error. The biggest error with the approach used is probably in the values for the cytosol fraction where especially broken chloroplasts would make a significant contribution of adenylates to this fraction. The ATP/ADP ratios for the cytosol should therefore be regarded as minimum values since in the situations studied the ATP/ADP ratios in the chloroplasts are relatively low.

In pioneering experiments by Heber and Santarius [13,14] the cytosolic ATP/ADP ratio was reported to be higher in the light than in the dark. In these experiments the leaves were rapidly killed by freezing and the cytosolic and chloroplastic ATP/ADP ratios determined after non-aqueous fractionation. Conflicting results were obtained by Stitt et al. [6] who showed with isolated protoplasts of wheat that under steady-state photosynthesis at high $CO_2$ concentration the cytosolic ATP/ADP ratio was lower in the light compared to the ratio in darkness. From the results in table 2 this discrepancy can easily be explained: the experiments using leaves were performed in air which gives a limiting supply of $CO_2$ as $CO_2$ is limiting for photosynthesis under normal atmospheric conditions for $C_3$ plants. The protoplast experiments by Stitt et al. [6] were on the other hand performed under conditions with saturating $CO_2$. It can thus be concluded that for $C_3$ plants under natural atmospheric conditions the cytosolic ATP/ADP ratio is higher in the light than in the dark. This has important implications for the regulation of many metabolic processes in the cytosol in the light (i.e. glycolysis and malate transport into the vacuole [15]).

It was originally suggested that the increased cytosolic ATP/ADP ratio in the light would inhibit mitochondrial respiration [14]. This might not be the case as very high extramitochondrial ATP/ADP ratios (>20) are needed to restrict respiration in isolated plant mitochondria [16]. Also, it seems from the data in table 3 that the gradient in ATP/ADP ratios between the cytosol and the mitochondrial matrix is reduced in the light both at limiting and high $CO_2$. As discussed by Stitt et al. [6] this would indicate a decreased energization of the mitochondria in the light, which is opposite to what would be expected if the electron transport was inhibited by a high cytosolic ATP/ADP ratio.

It is evident from the present study that it is an oversimplification to consider only dark versus light. The metabolic situation in light depends not only on the light intensity but many other factors including the $CO_2$ and $O_2$ concentrations.

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