Enzymic Determination of Metabolites in the Subcellular Compartments of Spinach Protoplasts

Received for publication November 26, 1979 and in revised form February 15, 1980

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ABSTRACT
A method for determining the subcellular metabolite levels in spinach protoplasts is described. The protoplasts are disrupted by centrifugation through a nylon net, releasing intact chloroplasts which pass through a layer of silicone oil into perchorlic acid while the remaining cytoplasmic components remain over the oil and are simultaneously quenched as acid is centrifuged into them. Cross-contamination is measured and corrected for using ribulose 1,5-bisphosphate as a chloroplastic marker and phosphoenolpyruvate carboxylase as a cytoplasmic marker. A method for separation of intact protoplasts from the medium by silicone oil centrifugation is described, which allows a correction to be made for the effect of free chloroplasts and broken protoplasts. Methods for inhibiting chloroplast photosynthesis, without inhibiting protoplasts, are presented. It is demonstrated that ribulose 1,5-bisphosphate, ATP, ADP, AMP, inorganic phosphate, hexose phosphate, triose phosphate, fructose 1,6-bisphosphate, and 3-phosphoglycerate can be reliably recovered in the subcellular fractions isolated from protoplasts, and measured by enzymic substrate analysis.

Isolated intact chloroplasts are widely used to investigate photosynthesis. However the isolation of chloroplasts can produce changes in their metabolism. Interactions with the cytoplasm and other organelles are lost, and changes in the rates of transport of metabolites across the bounding envelope membrane are to be expected. Moreover, important processes which occur in the cytoplasm and other organelles cannot be studied; these include sucrose synthesis, interactions with respiration, photosynthesis, and aspects of nitrogen metabolism and lipid metabolism. On the other hand, isolated cells (15) or protoplasts (4) provide an easily manipulable whole cell system but pose the problem of resolving the internal compartmental organization.

When protoplasts are forced through a nylon net, a protoplast extract is produced, in which the chloroplasts are still intact (4). The possibility emerged that protoplasts could be centrifuged through a net held in a centrifuge tube and the resulting intact chloroplasts immediately separated from other components because they would pass through a layer of silicone oil. Recently, Robinson and Walker (20) have shown the feasibility of this approach by studying the separation of enzymes diagnostic of the various subcellular compartments. Parallel work has proceeded in our laboratory to investigate whether this method can be used to give reliable measurements of the subcellular metabolite levels. The method has been specifically developed for the enzymic analysis of the subcellular levels of metabolites in unlabeled extracts but can obviously be extended to, and used in conjunction with, radioactive analysis techniques.

A method for subcellular metabolite analysis must meet the following criteria. (a) The cellular compartments must be separated into fractions with acceptably low levels of cross-contamination. The extent of cross-contamination must be determined and corrected for by the use of suitable markers. (b) Separation and quenching of the fractions must be rapid enough to prevent any significant changes in the metabolite levels during, or as a result of, the fractionation procedures. (c) Protoplast preparations inevitably contain some free chloroplasts. The activity of these chloroplasts must be inhibited, without altering protoplast metabolism. The end products of chloroplast photosynthesis which accumulate in the medium are triose-P and, to a lower extent, 3-P-glycerate (2, 13). These are relatively small, rapidly turning-over intermediate pools in protoplasts. (d) Analytic techniques must be developed to permit recovery and measurement of the intermediates.

MATERIALS AND METHODS
Spinach (Spinacia oleracea L. U.S. Hybrid 424, Ferry-Morse Seed Co.) was grown in water culture (12). [14C]Sorbitol, [14C]NaHCO₃, and [3H]H₂O were obtained from Amersham, Braunschweig. Biochemicals and enzymes were from Boehringer Mannheim, with the exception of the high specific activity invertase, obtained from Sigma. Biochemical grade sorbitol (Merck, Darmstadt) was used throughout. Metrizamide was from Sigma, and Percoll was from Pharmacia.

Protoplast Preparation. Protoplasts were prepared from 4- to 5-week-old spinach leaves according to Edwards et al. (4), with modifications. The upper epidermis was removed so that predominantly palisade cell protoplasts were isolated. Prepared leaf pieces were floated on 0.5 M sorbitol, 1 mM CaCl₂, 5 mM Mes (pH 5.6), 0.05% (w/v) BSA, and 0.05% (w/v) PVP at 0-4°C until sufficient tissue was obtained; the solution was then replaced with 40 ml of a similar one containing, additionally, 2.5% (w/v) Cellulose Onuzuka SS and 0.5% (w/v) Maceroenzyme R-10 (Yakult Enz. Co. Ltd., Nishinomiya, Japan), and the leaf pieces incubated for 3.5 h at 28°C, while illuminated by a 150-W projector lamp (180 × 10⁹ erg cm⁻² s⁻¹). After careful removal of the enzyme solution, the leaf pieces were gently agitated with three successive 20-ml aliquots of 0.5 M sorbitol, 5 mM Mes (pH 6.0), 1 mM CaCl₂, 0.05% (w/v) BSA, and 0.05% (w/v) PVP, which were then combined and passed successively through a tea sieve and a 200-μm net, centrifuged for 5 min at 100g (800 rpm, Heraeus Christ Labofuge I), the supernatant decanted and the pellet gently resuspended in the residual fluid. The protoplasts were then purified on a metrizam-
ide-sorbitol isosmotic gradient consisting of three phases. The lower phase III contained the resuspended pellet in a final volume of 4.9 ml, 92 mM metrizamide, 0.408 mM sorbitol, 5 mM Mes (pH 6.0), and 1 mM CaCl₂. Layered above were 2 ml phase of II, containing 75 mM metrizamide, 0.425 mM sorbitol, 5 mM Mes (pH 6.0), and 1 mM CaCl₂, and 1 ml phase of I, containing 0.5 mM sorbitol, 5 mM Mes (pH 6.0), and 1 mM CaCl₂. The gradient was centrifuged for 7 min at 250g (1,200 rpm in a Heraeus Christ Labofuge I), the protoplasts collected from the interface of phase I and II and diluted with phase I to 200 μg Chl/ml. The preparation and storage of protoplasts were carried out at 0–4°C.

Unless specified otherwise, all experiments with protoplasts were carried out with a suspension diluted to give 50 μg Chl/ml in 0.41 mM sorbitol, 1.25 mM Mes, 20 mM Hapes (pH 7.6), 7.5 mM NaHCO₃, and 7.5 mM CaCl₂. Illumination was from a 150-W projector lamp (80 × 10⁵ ergs cm⁻² s⁻¹) and the temperature was 20°C.

**Silicone Oil Centrifugation of Intact Protoplasts.** Protoplasts were separated from their suspending medium by silicone oil centrifugation. Microfuge tubes (400 μl, Sarstedt) containing 40 μl of 15% HClO₄, 70 μl silicone oil (75 ml AR 20 and 180 ml AR 200, Wacker Chemie, München), and 100 μl protoplasts were centrifuged for 6 s in a Beckman microfuge 152. Immediately after the centrifuge came to rest, 85-μl aliquots of the supernatant were added to 12.5 μl dimethylformamide (pH 12.5). The tube was cut through the silicone oil layer, the protoplast pellet and HClO₄ removed from the tip, and combined with 80 μl water used to rinse the tip interior. All samples were centrifuged (2 min, Eppendorf microfuge) to remove precipitated protein, neutralized with 5 mM KOH, 1 mM triethanolamine, and again centrifuged to remove the HClO₄. Usually four to 12 tubes were used per sample; the extracts were combined before neutralization.

**Subcellular Fractionation of Protoplasts.** Protoplasts were fractionated into chloroplast and extrachloroplast components (Fig. 1) in polypropylene microfuge tubes (Sarstedt, 400-μl volume) which contained, successively, 20 μl of 10% HClO₄, 70 μl silicone oil (19 ml AR 200 and 1 ml AR 20), and air space of approximately 130 μl, and a 17-μm net (PE 17 HD, Züricher Beuteltuchfabrik AG, Postbach, CH-8803 Rüschlikon, Switzerland) held securely in place by a 14 mm length of polypropylene tubing (3 × 4 mm; width, 0.5; mm; Bender and Hobein GmbH, Postbach 150 229, München 15). The tubing is used to force the net down into the microfuge tube, so that the entire net periphery is tightly clamped between the tube and the microfuge tube. Protoplasts, 100 μl, were pipetted into the space over the net. A lid, containing 10 ml 0.3 N HCl in the well, was fitted over the microfuge tube. A fine pore connected the well of the lid with the inner surface of the lid. The tube was centrifuged for 9 s in a Beckman microfuge 152 at 20°C. Immediately after centrifugation, a 90-μl aliquot of the extrachloroplast supernatant was deproteinized with 12.5 μl of 60% (v/v) HClO₄. The chloroplast pellet was recovered and all fractions neutralized as described already.

**Enzyme Measurement.** NADP-glyceraldehyde-3-P dehydrogenase (EC 1.2.1.9) and PEP³ carboxylase (EC 4.1.1.31) were assayed as in Stitt et al. (23).

**Analysis of Metabolites.** RuBP was determined by 14C incorporation in the presence of RuBP carboxylase. Extract, equivalent to 1.5–2 μg Chl, was incubated for 30 min at 20°C in a final volume of 250 μl with 100 mM Tris-HCl (pH 8.1), 20 mM MgCl₂, 2 mM NaHCO₃, 1 mM dicyclohexyl-phenol (included to inhibit Prlribokinase present in the RuBP carboxylase preparation), 4 μCi [1-14C]NaHCO₃, and 50 ng purified RuBP carboxylase. Then 200-μl aliquots were added to 500 μl 10 N HCOOH, evaporated to dryness at 50–65°C, reconstituted in 100 μl 1 N HCl, reevaporated, and added to scintillation fluid (6 g butyl-PBD, 600 ml toluene, 400 ml methylglycol). RuBP carboxylase was prepared as described in Pausen and Lane (16). Immediately before use it was freed from (NH₄)₂SO₄ by centrifugation, resuspended in 100 mM Tris-HCl (pH 8.1), and passage through a Sephadex G-25 column equilibrated with Tris-HCl (pH 8.1).

Glc-6-P, Fru-6-P, and Glc-1-P were measured spectrophotometrically (14). Triose-P, Fru-1,6-bisP and 3-P-glycerate were measured spectrophotometrically by a method modified from Lowry and Passoneau (14). To extract, in 50 mM imidazole-HCl (pH 7.1), 20 mM NaCl, 1 mM MgCl₂, 2 mM mercaptoethanol, 1 mM ATP, and 400 μM NADP were added, successively, 0.3 U glycerol-3-P dehydrogenase, 5 U triose-P isomerase, 0.1 U aldolase, and 0.9 U P-glyceraldehyde kinase with 0.8 U glyceraldehyde-3-P dehydrogenase. All enzymes, apart from glycerol-3-P dehydrogenase, were desalted by centrifugation immediately before use. This method of assay economized on the volume of extract required and oxidized 2 NADH molecules per 3-P-glycerate present. Sucrose was measured spectrophotometrically (10).

Pi was measured spectrophotometrically in a new two step method which increases sensitivity and decreases nonextractable blanks deriving from biochemicals and enzymes (14). Equal volumes, 100 μl, of extract and of 100 mM imidazole-HCl (pH 7.0) with 1.2 mg/ml glycogen, 100 μM AMP, 5 μM Glc-1,6-bisP, 10 mM EDTA, 5 mM Mg-acetate, 5 mM DTT, 0.14 U phosphorylase, and 0.7 U P-glucomutase were incubated 1.5 h at 20°C, boiled for 2 min, and the combined Glc-6-P and Glc-1-P then measured, as above, in a volume of 1,000 μl. The glycogen had been dialyzed 12 h against two changes of a 50-fold excess of 100 mM imidazole-HCl (pH 7.0). Phosphorylase and P-glucomutase were dialyzed against three successive 50-fold excess volumes of 100 mM imidazole-HCl (pH 7.0), each for 45 min. This method gave nonextract blank values of 0.1–0.3 nmol Pi/assay and linear standard curves from 0.3–50 nmol Pi/assay. Of the added Pi, 75–90% was recovered, the remainder could be accounted for by the equilibrium between free Pi, glycogen, and Glc-1-P.

Photometric analysis was performed with a Hitachi/Perkin-Elmer 156 Double Beam Spectrophotometer, or a Perkin-Elmer 556 Double Beam Spectrophotometer, with assay volumes of 600–1,000 μl.

Adenine nucleotides were determined by the luciferase method. For measurement of ADP and AMP, the 1 TmP and buffer were pretreated with activated charcoal, to decrease the blank values. Sorbitol and titrated water spaces were measured and calculated as in reference 8.

³ Abbreviations: PEP: phosphoenolpyruvate; RuBP: ribulose 1,5-bis-phosphate; Glc-1,6-bisP: glucose 1,6-bisphosphate; Glc-6-P: glucose 6-phosphate; Glc-1-P: glucose 1-phosphate; Fru-6-P: fructose 6-phosphate; Fru-1,6-bisP: fructose 1,6-bisphosphate.

![Fig. 1. Rapid separation and immediate quenching of chloroplast and extrachloroplast fraction from spinach protoplasts.](image-url)
RESULTS AND DISCUSSION

Preparation of Protoplasts. As one of the aims of this method is to study the synthesis and distribution of sucrose, it was neces-
sary to purify protoplasts by a method other than a sucrose
gradient (4). Isosmotic gradients with Percoll and metrizamide
proved suitable; for experiments described here, a metrizamide
density gradient was used. Metrizamide did not have detrimental
effects on the protoplasts. A batch of unpurified protoplasts was
divided; half was purified with a sucrose gradient and half with a
metrizamide gradient. The photosynthetic rates of the protoplasts
so obtained were similar, or even higher for the metrizamide
gradient-isolated protoplasts. Under certain conditions the pres-
ence of sucrose may produce a substantial inhibition of photosyn-
thesis (Stitt, unpublished). Protoplasts were prepared from pali-
side cells because they had higher metabolite levels and rates of
photosynthesis than spongy mesophyll cells. Visual inspection also
showed that most of the leaf starch is confined to the palisade
layer. The protoplasts isolated from spinach palisade cells had
high rates of photosynthesis (139 ± 18 µmol O₂ mg⁻¹ Chl h⁻¹) and
synthesized starch.

Subcellular Fractionation of Protoplasts. The principle of the
method is illustrated diagrammatically in Figure 1. The pro-
toplasts, initially held above a nylon net, are centrifuged
through the net, disrupting them, and releasing intact chloroplasts
which immediately continue downwards through the silicone oil into the
10% HClO₄ where their metabolism is quenched. This fraction is
denoted F1. The extrachloroplastic components, set free by the
disruption of the protoplasts, remain over the oil. The HCl drop-
et is forced through the fine pore in the lid and mixes with the
extrachloroplastic supernatant, lowering the pH to 2.5–3.5 and so
halting metabolism. This preliminary acidification is rapid, but
not so rapid that the intact chloroplasts can not enter the silicone
oil before they are affected. Immediately after centrifugation, an
aliquot of the extrachloroplast supernatant is deproteinized with
HClO₄, giving fraction F2.

In experiments where the distribution of enzymes was studied,
the 10% HClO₄ under the silicone oil was replaced with 25% (w/ w)
sucrose, 27 mΜ Heps (pH 7.6), 7.5 mΜ CaCl₂, and the HCl
droplet in the lid was omitted.

The mechanical details of the technique described in this paper
resemble those of the method developed by Robinson and Walker
(20) in that an air space is included between the secured net and
the silicone oil. The volume of the air space is greater than that of
the protoplast suspension, allowing the entire mass to pass through
the nylon net instantaneously. This facilitates rapid disruption of
the protoplasts and separation of the fractions. The two methods
differ in technical details of how the net is held in place and where
the protoplasts are located. The method of Robinson and Walker
allows more protoplasts to be handled per tube, but the present
method does not require modifications to the centrifuge and the
tubes can be very rapidly assembled. It also provides a means of
halting metabolism in the extrachloroplastic supernatant.

Measurement of Cross-Contamination. Initially, separation
was monitored by marker enzymes for the chloroplast and cytosol,
NADP-glyceraldehyde-3-P dehydrogenase and ATP-cytochrome
oxidase, respectively (23). In seven to 15 experiments, 81 ± 2% of the
NADP-glyceraldehyde-3-P dehydrogenase and 2.6 ± 0.5% of the
PEP carboxylase were present in the chloroplast pellet (F1). Of
the activity in the whole protoplasts, 102 ± 3% and 104 ± 5% of
the activity of these two enzymes was recovered in the summed
fractions, F1 + F2. Most of the NADP-glyceraldehyde-3-P dehy-
drogenase was already present in the pellet after 3 s.

Subsequently, in experiments studying substrate compartment-
tation, RuBP was used as a chloroplast marker. It reflects more
accurately the cross-contamination by substrates than would an
enzyme, whose retention in partly damaged chloroplasts may be
greater. Also, by using RuBP, the cross-contamination in the
individual samples destined for substrate analysis can be separ-
ately determined. PEP carboxylase was retained as a cytosolic
marker. Sucrose showed an anomalously high percentage in the
chloroplast pellet (F1) suggesting a complicated localization. Amino
acids (11), organic acids (24), and the unique glycolytic intermediates (24) are found in the chloroplasts and, hence, are
unsuitable for a cytosolic marker. The distribution of mitochondri-
a and other organelles has not been studied. Published data
(20) suggest that they remain largely above the silicone oil.

Metabolite Levels are Unperturbed by Fractionation. Two
experiments were carried out to show that metabolite levels are not
altered when protoplasts are centrifuged, disrupted and separated
into chloroplast and extrachloroplast fractions, and that the me-
tabolites are completely recovered through the processes of
quenching, extraction, storage, neutralization, and assay.

The following experiment demonstrates that the fractionation
of the protoplasts does not lead to significant changes in the
metabolite levels in the protoplasts. Some protoplasts were killed
by immediate addition of HClO₄ to the suspension. Parallel
samples were fractionated to give chloroplast (F1) and extracho-
loroplast (F2) fractions. The metabolites in the chloroplast and
extrachloroplast were measured, summed, and compared with the
total amounts found in parallel samples of protoplasts which were
killed without fractionation by directly adding HClO₄ to the
protoplast suspension (Table 1). No large differences were found
for any substrate, showing that detectable alterations are not
produced by the fractionation procedures. The complete recovery,
and the fact that the se was mostly under 7% of the mean, also
demonstrates the accuracy of the metabolite analysis techniques
with these amounts of substrates. The se was quite large for AMP,
and the recovery was not as satisfactory as for the other metab-
olites. The AMP level is calculated from the difference between
the total adenine nucleotides, and the sum of the ATP plus ADP. The
ATP plus ADP usually represent the major part of the total
adenine nucleotides, hence some error is inherent in the calcula-
tion.

The adequacy of the quenching of the fractions, the storage
conditions, and the assay techniques were demonstrated by show-
that small, representative quantities of biochemicals added to

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Summed Metabolite Level in Chloroplast and Extrachloroplast Fraction on a Percentage of Metabolites in Protoplast Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-1-P</td>
<td>100 ± 11.5</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td>99 ± 5.5</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>117 ± 6.5</td>
</tr>
<tr>
<td>Fru-1,6-bisP</td>
<td>99 ± 15</td>
</tr>
<tr>
<td>Triose-P</td>
<td>103 ± 3.0</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>110 ± 11.0</td>
</tr>
<tr>
<td>RuBP</td>
<td>103 ± 2.5</td>
</tr>
<tr>
<td>ATP</td>
<td>104 ± 3.3</td>
</tr>
<tr>
<td>ADP</td>
<td>109 ± 3.4</td>
</tr>
<tr>
<td>AMP</td>
<td>130 ± 12.5</td>
</tr>
<tr>
<td>SUM + ADP + ATP</td>
<td>99.2 ± 6.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>103 ± 11.1</td>
</tr>
<tr>
<td>Pi</td>
<td>102 ± 1.0</td>
</tr>
</tbody>
</table>
the different fractions could be quantitatively recovered and measured (Table II). The biochemicals were added in amounts similar to those present endogenously in the fractions. They were added as soon as physically possible after the protoplasts had been disrupted, so that these added biochemicals were exposed to the same conditions as the endogenous substrates (Table II). No large losses of any biochemical were found.

The acidification of the supernatant during centrifugation might lead to the loss or modification of the chloroplasts if they do not enter the oil rapidly enough. Two experiments exclude this possibility. First, the proportion of chloroplasts recovered in the pellet is not decreased by acidification of the supernatant. In three separate fractionations during which the supernatant was simultaneously acidified, 80 ± 7 and 78 ± 5% of the glyceraldehyde-3-P dehydrogenase and RuBP were recovered in the chloroplast sediment (F1). Corresponding values when the acidification was omitted were 74 ± 3 and 79 ± 8, respectively. Second, the metabolite levels in the chloroplast pellet (F1) were compared in the parallel samples which had been centrifuged with, and without, the simultaneous addition of HCl (Table III). No changes were found. If the chloroplasts were affected by the acidification then an inhibition of photosynthesis should result from acidification of the stroma (19), resulting in an increase in triose-P, Fru-1,6-bisP, and ATP, and decreases in 3-P-glycerate and ADP.

The percentage of the PEP carboxylase in the chloroplast sediment (F1) was similar when HCl was added simultaneously to the supernatant (3 ± 0.5) or omitted (4 ± 1), so cross-contamination from the cytosol is not increased by simultaneous acidification. However, without acidification, the metabolite levels in the extrachloroplast supernatant (F2) itself did change (experiments not shown). In particular, most of the Fru-1,6-bisP was lost and

Table II. Recovery of Substrates Added to Chloroplast and Extrachloroplast and Medium Fraction

<table>
<thead>
<tr>
<th>Added Substrate</th>
<th>Chloroplast Fraction, F1</th>
<th>Extrachloroplast Fraction, F2</th>
<th>Protoplast Suspension Medium, F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-6-P</td>
<td>107</td>
<td>101</td>
<td>87</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>106</td>
<td>103</td>
<td>81</td>
</tr>
<tr>
<td>Fru-1,6-bisP</td>
<td>98</td>
<td>103</td>
<td>102</td>
</tr>
<tr>
<td>Triose-P</td>
<td>116</td>
<td>103</td>
<td>100</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>96</td>
<td>98</td>
<td>86</td>
</tr>
<tr>
<td>ATP</td>
<td>101</td>
<td>82</td>
<td>78</td>
</tr>
<tr>
<td>ADP</td>
<td>122</td>
<td>105</td>
<td>108</td>
</tr>
<tr>
<td>ATP + ADP + AMP</td>
<td>96</td>
<td>84</td>
<td>76</td>
</tr>
<tr>
<td>RuBP</td>
<td>110</td>
<td>111</td>
<td>85</td>
</tr>
<tr>
<td>Pi</td>
<td>99</td>
<td>93</td>
<td>102</td>
</tr>
</tbody>
</table>

Table III. Metabolite Levels in the Chloroplast Fractions Are Not Altered by Acidification during Fractionation of Protoplasts

Protoplasts were incubated for 2 min in the dark and 2 min in the light at 20°C and then fractionated. The supernatant was acidified during fractionation as described in "Materials and Methods". The fractions were collected, neutralized and assayed as described. Each sample contained at least eight separately fractionated 100-μl volumes of protoplasts. Each value is the mean of two or three experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount of Substrate in Chloroplast Fraction (F1) Separated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Instantaneous Acidification of the Supernatant</td>
</tr>
</tbody>
</table>
| Glc-1-P   | 5 | 3 | 2
| Glc-6-P   | 24 | 22 | 18
| Fru-6-P   | 11 | 11 | 8
| Fru-1,6-bisP | 18 | 18 | 15
| Triose-P  | 25 | 25 | 15
| 3-P-glycerate | 47 | 50 | 11
| RuBP      | 81 | 80 | 8
| ATP       | 15 | 15 | 11
| ADP       | 11 | 14 | 11
| AMP       | 11 | 11 | 11

Table IV. Effect of CaCl₂, Pi, and pH on Photosynthesis of Protoplasts and Chloroplasts

Palisade protoplasts were prepared. Protoplast extracts were prepared from the protoplasts by three passages through a 17-μm nylon net on the end of a syringe. Photosynthesis was measured in an O₂ electrode at 20°C. Illumination was with a white light from a 150-w projector lamp, 200 x 10⁻¹⁰ erg cm⁻² s⁻¹ for protoplasts; for protoplast extract, a red filter was included. The assay was in 0.4 mM sorbitol, 25 mM Hepes (pH 7.6), and 10 mM NaHCO₃, or 0.4 mM sorbitol, 25 mM Mes (pH 6.5), and 1.2 mM NaHCO₃. Other additions are specified in the table.

<table>
<thead>
<tr>
<th>pH</th>
<th>7.5 mM CaCl₂</th>
<th>2.5 mM KH₂PO₄</th>
<th>O₂ Evolution from Protoplasts and Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protoplasts</td>
<td>Extract</td>
<td>μmol mg⁻¹ Chl h⁻¹</td>
</tr>
<tr>
<td>7.6</td>
<td>−</td>
<td>−</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>68</td>
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<td>78</td>
</tr>
<tr>
<td>6.5</td>
<td>−</td>
<td>−</td>
<td>77</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>72</td>
</tr>
</tbody>
</table>

Table IV. Effect of CaCl₂, Pi, and pH on Photosynthesis of Protoplasts and Chloroplasts

There were corresponding increases in the triose-P and hexose-P. Fru-1,6-bisP has single substrate reactions with both aldolase and Fru-1,6-bisPase. The rates of such reactions would not be decreased as much by dilution as are the rates of reactions with two or more substrates. In addition, the proportion of the Fru-1,6-bisP hydrolyzed by aldolase at equilibrium is strongly concentration dependent.

Inhibition of Photosynthesis of Free Chloroplasts. Three methods were developed to restrict chloroplast photosynthesis without inhibiting protoplast photosynthesis (Table IV). Addition of 2.5 mM KH₂PO₄, 7.5 mM CaCl₂, or lowering the pH to 6.5 inhibit photosynthesis of the chloroplast in a protoplast extract. None of these treatments impair protoplast photosynthesis. For example, when chloroplast photosynthesis was inhibited by low pH or 7.5
mm CaCl₂, addition of KH₂PO₄ produces no further decrease in the rate of CO₂-dependent O₂ evolution. At pH 7.6 in the absence of CaCl₂, when some chloroplast photosynthesis is possible, KH₂PO₄ did decrease the overall rate of CO₂-dependent O₂ evolution by a protoplast suspension. The possibility that some components of the photosynthesis observed with protoplasts might be due to free chloroplasts should always be critically examined.

Rapid photosynthesis by protoplasts at pH 6.5 could be achieved only when the bicarbonate concentration was lowered. The concentration of CO₂ in equilibrium with a given bicarbonate concentration rises as the pH becomes more acid. High CO₂ inhibits photosynthesis, catalyzing a proton shuttle across the envelope membrane (7) and preventing the light-dependent alkalinization of the stroma. Provided only traces of bicarbonate are supplied, rapid photosynthesis can be obtained by protoplasts in a pH 5.0 medium. The precise effects of CaCl₂ are not fully understood, but it also enhances (Stitt, unpublished) protoplast stability and photosynthesis.

In this method, 7.5 mm CaCl₂ was selected to inhibit chloroplast photosynthesis. The use of low pH is a viable alternative. The use of high Pi was not applied, as it might cause an exchange of metabolites out of the chloroplasts, via the phosphate translocator, in the brief period between disruption of the protoplasts and entry of the chloroplast into the silicone oil.

Separation of Intact Protoplasts from the Suspending Medium. Even when the rates of chloroplast photosynthesis are very low, the resulting production of triose-P and 3-P-glycerate must still be taken into account. For example, if only 10% of the protoplasts have broken to release chloroplasts which photosynthesize at the very low rate of 5 mmol CO₂ mg⁻¹ Chl h⁻¹, they would produce about 11 nmol triose-P in 4 min. This is half the size of the total cytoplasmic pool. The products of chloroplast photosynthesis are exported to the medium (2, 13) and will, therefore, be included in the metabolite levels in the extrachloroplasmic supernatant (F2). Unless corrected for, they would give an overestimate of the metabolites present in the cytosol of the protoplasts. Similarly low levels of metabolites released into the medium from protoplasts which have broken during storage will produce an overestimate of cytosolic metabolite levels.

Therefore, the metabolite levels in the medium were determined, and the results for the extrachloroplasmic supernatant (F2) accordingly corrected by subtraction. A suspension containing intact protoplasts was layered over silicone oil and centrifuged, giving a sediment (F4), corresponding to the intact protoplasts, and a supernatant (F3), corresponding to the medium in which the protoplasts were suspended. The reliability of the correction depends upon having recovered the metabolites which were originally in the medium, and upon having separated the protoplasts from the medium without breaking a large proportion during the centrifugation and releasing their contents into the medium.

The following experiment demonstrates that marked perturbations of metabolites do not occur in the medium as a result of the fractionation procedures. Representative amounts of metabolites, in a 5-μl droplet at the mouth of the centrifuge tube, were centrifuged into the supernatant medium (F3) while the protoplasts were centrifuged out through the silicone oil (Table II). The metabolite levels in these samples, and in control samples without added metabolites, were determined and the difference between them expressed as a percentage of the added metabolite to give the recovery of added metabolite. Recovery was over 80% in all cases.

If many of the protoplasts broke during centrifugation and released their contents back into the medium then the metabolite levels found in the medium supernatant fraction (F3) would be an overestimate of the levels really present in the medium during the incubation. Hence the correction of the extrachloroplast fraction would be too large. Three lines of evidence argue that the protoplasts enter the oil without serious breakage. First, when protoplasts were separated from the medium by silicone oil centrifugation, 93% or more of the total fixed carbon was present in the protoplast sediment (F4) after 0–2 min photosynthesis. Second, protoplasts layer above 25% (w/w) sucrose while chloroplasts sediment through it. After centrifuging protoplasts through silicone oil into 20 μl of 25% (w/w) sucrose the vast majority of the Chl layered at the oil/sucrose interphase and microscopic examination revealed intact protoplasts. Third, only 13–20% of the total hexose-P, as measured by enzyme analysis, was found in the medium fraction (F3). The percentage of the protoplasts broken during centrifugation will be lower, as some of the hexose-P derives from previously broken protoplasts.

Silicone oil centrifugation of intact protoplasts was used to compare the sorbitol and tritiated water spaces of protoplasts and the chloroplasts derived from them (Table V). In chloroplasts, the sorbitol-impermeable tritium permeable space is the region within the inner envelope membrane. Besides the adherent medium, the sorbitol-permeable space is, in large part, the region between the inner and outer envelope membranes. Consequently the sorbitol space is relatively large (8). In protoplasts the sorbitol space is small. Sorbitol will be excluded by the plasmalemma, and there is no external sorbitol-permeable membrane space to trap medium. But the tritium-permeable, sorbitol-impermeable space is far larger in protoplasts than chloroplasts. It represents, in terms of the Chl, not only the chloroplast volume but also the cytoplasm, other organelles, and vacuole. It should also be noted that only 0.1 or 0.2% of the medium volume is carried through the silicone oil with protoplasts (or chloroplasts). Thus the cross contamination between the free medium and the protoplasts (F4) or chloroplasts (F1) fractions is negligible.

**Table V. Measurement of Sorbitol and Tritium Spaces in Protoplasts and Protoplast Extracts**

<table>
<thead>
<tr>
<th>Space</th>
<th>μl/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol space</td>
<td>20</td>
</tr>
<tr>
<td>H₂O space</td>
<td>283</td>
</tr>
<tr>
<td>Sorbitol-impermeable H₂O space</td>
<td>263</td>
</tr>
<tr>
<td>Sorbitol space</td>
<td>0.08</td>
</tr>
<tr>
<td>Sorbitol impermeable space</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Protoplasts were incubated and fractionated (below). After incubation with 10 μCi [³H]H₂O and 1 μCi [¹⁴C]sorbitol at 4 C for 3 min, the radioactivity in the resulting supernatant and pellet were determined. The percentage of supernatant carried through with the pellet is calculated by multiplying the Chl per tube by the sorbitol space, dividing by the total volume of the supernatant and multiplying by 100.
recovered in F1 are calculated:

\[ a = \frac{\text{RuBP}_2 - \text{RuBP}_3}{\text{RuBP}_1 + \text{RuBP}_2 - \text{RuBP}_3} \]

\[ b = \frac{\text{PEP carboxylase}_1}{\text{PEP carboxylase}_1 + \text{PEP carboxylase}_2 - \text{PEP carboxylase}_3} \]

Values for \( a \) vary from 0.32 to 0.15 and can be determined individually for each F1 and F2 sample pair. A value of \( b = 0.024 \) was taken from the mean of 15 determinations. The following equations can be written and solved for \( C \) and \( E \):

\[ C + E = A1 + A2 - A3 \]

\[ A1 = (1 - a) \cdot C + b(A2 - A3) \]

\[ C = \frac{A1 - b(A2 - A3)}{(1 - a)} \]

\[ E = A1 + A2 - A3 - c \]

The calculation assumes that cross-contamination from the metabolites in the medium (F3) in the chloroplast fraction (F1) is negligible. Only 0.2% of the medium is carried through the silicone oil with the chloroplasts when a protoplast extract is centrifuged (Table VI). This is over 10 times lower than the cross-contamination for the cytosolic PEP carboxylase, 2.4 ± 0.5%. The results in Table VI represent a typical fractionation of protoplasts, yielding F1 and F2, and separation of protoplasts from the suspending medium, yielding F3 and F4. The protoplasts had been given 2 min light. The metabolites present in the intact protoplasts, F4, are included for comparison with the amounts calculated to be present in the protoplasts using the other three fractions (F1 + F2 - F3). The agreement is good. The calculated metabolites present in the chloroplast (C) and extrachloroplast (E) compartments of the cell are given in the last two columns. Although in large part the corrected results resemble the results of the measurements of the fractions, the importance of the corrections is clearly shown. Ignoring the effect of metabolites in the medium would lead to significant errors in the extrachloroplast metabolite levels. The calculations of cross-contamination between F1 and F2 also produce important corrections, as in for example, the cytosolic Fru-1,6-bisP levels.

Some inaccuracy in the corrections must occur if a metabolite is almost entirely in one compartment. For example, almost all the phosphate is in the extrachloroplast compartment. Thus a significant proportion of the total Pi in F1 is due to cross-contamination. If the correction for cross-contamination is a little too small, or large, the calculated absolute amount of Pi in the chloroplasts will be inaccurate, even though the relative amounts in a series of samples can still be usefully compared.

**Intracellular Distribution of Metabolites.** Some results of interest emerge from this single experiment. The ratio of 3-P-glycerate to triose-P is much higher in the chloroplast (3.77) than in the extrachloroplast (0.51) compartment. Correspondingly, the relative chloroplastic to extrachloroplastic content is far higher for 3-P-glycerate (3.80) than for triose-P (0.52). This is reminiscent of the situation in the isolated chloroplast (13) and indicates that triose-P is preferentially exported from the chloroplast in situ in the light. The ATP/ADP ratio is higher in the extrachloroplast than in the chloroplast compartment. Similar results have been obtained from nonaqueously fractionated leaves (6). The difference may reflect the rapid use of ATP by the Calvin cycle and the exact coupling of the adenylate pools to redox couples and transport systems. It is also noteworthy that the relative amounts of Fru-1,6-bisP and dihydroxyacetone-P differ greatly between the chloroplast (1.76) and extrachloroplast compartments (0.08). Assuming equal volumes of the chloroplast and cytoplasm and that dihydroxyacetone-P and glyceraldehyde 3-P are in a 10:1 ratio, the calculated mass action ratios from the data in Tables V and VI are 2.5 × 10⁻² (chloroplast) and 1 × 10⁻³ (cytoplasm). The equilibrium constant for the aldolase reactions varies from 1.9 × 10⁻¹ to 1.4 × 10⁻⁴ as the Mg increases from zero to 10 mM (3). The chloroplast and cytosol-free Mg is probably within this range; thus, it seems the aldolase reaction is slightly removed from equilibrium in opposing directions in the chloroplast and cytosol.

Considerable hexose-P is found in both the chloroplast and extrachloroplast compartments. The protoplasts contain substantial sucrose, with an average concentration of 1.9 mM. If this were all in the cytosol, a concentration of 10–15 mM would result. The precise location of the sucrose would merit further attention, both with reference to its interactions with photosynthesis and its export, or storage, in the mesophyll cells. The reason why a much higher percentage of the total sucrose is found in the chloroplast fraction than in the case for PEP carboxylase, or even phosphate, is unclear.

The over-all content of Pi in protoplasts is substantial. Of this, only about 4% of the total Pi was found in the chloroplast. High over-all levels of Pi and a similar low proportion in the chloroplasts were also found in nonaqueously fractionated leaves (21) and it was suggested that much of the Pi might be in the vacuole (25). In these spinach protoplasts the average Pi concentration was 23 mM. If all the extrachloroplastic Pi were in the cytosol a concentration of about 150 mM can be calculated, assuming that the cytosol volume is similar to the chloroplast volume (47 µl/mg Chl). Such a high concentration is difficult to reconcile with the far lower

Table VI. *Substrate Analysis in Spinach Protoplasts*

<table>
<thead>
<tr>
<th>Fractionated Protoplasts</th>
<th>Intact Protoplasts</th>
<th>Metabolites in</th>
<th>Chloroplasts</th>
<th>Extrachloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F₁ Sediment</td>
<td>F₂ Supernatant</td>
<td>F₃ Supernant</td>
<td>F₁, F₂, F₃</td>
</tr>
<tr>
<td>Pi</td>
<td>349</td>
<td>11,060</td>
<td>5,510</td>
<td>6,030</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>50.7</td>
<td>73.6</td>
<td>42.2</td>
<td>89.7</td>
</tr>
<tr>
<td>Triose-P</td>
<td>14.5</td>
<td>79.2</td>
<td>42.8</td>
<td>44.5</td>
</tr>
<tr>
<td>Fru-1,6-bisP</td>
<td>23.9</td>
<td>9.6</td>
<td>0</td>
<td>36.9</td>
</tr>
<tr>
<td>RuBP</td>
<td>17.0</td>
<td>6.1</td>
<td>1.2</td>
<td>21.5</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td>29.7</td>
<td>97.7</td>
<td>36.9</td>
<td>81.2</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>13.0</td>
<td>34.3</td>
<td>13.2</td>
<td>29.7</td>
</tr>
<tr>
<td>ATP</td>
<td>12.0</td>
<td>21.4</td>
<td>3.2</td>
<td>26.4</td>
</tr>
<tr>
<td>ADP</td>
<td>8.1</td>
<td>13.6</td>
<td>3.1</td>
<td>18.3</td>
</tr>
<tr>
<td>AMP</td>
<td>10.9</td>
<td>11.3</td>
<td>4.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>79</td>
<td>707</td>
<td>258</td>
<td>451</td>
</tr>
</tbody>
</table>

nmol/mg Chl

5,900 238 5,670

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concentrations of the other substrates for the phosphate translocator in the cytosol and chloroplast. A problem is also posed with regard to starch synthesis if such amounts of Pi are readily available to metabolism. In these protoplasts starch synthesis was reasonably rapid, accounting for 10 to 20% of the total photosynthetic (Stitt, Wirtz, and Heldt, in preparation). However, it has been shown in leaves (9) and isolated chloroplasts (9, 18, 22) that starch synthesis is initiated by Pi depletion. Further experiments are required to establish the precise location of the Pi.

Comparison of Chloroplast Metabolite Levels in Vitro and in Situ. The published data for the levels of metabolites found in photosynthesizing isolated chloroplasts can be compared with those found in chloroplasts photosynthesizing in situ in protoplasts (Table VII). Similar levels are found for 3-P-glycerate, RuBP, Pi, and hexose-P in isolated chloroplasts and in chloroplasts contained in protoplasts. However, the Fru-1,6-bisP and triose-P are both three to seven times higher in the chloroplasts from protoplasts and, as a result (17) the ATP/ADP quotient is altered. The lower triose-P and Fru-1,6-bisP in isolated chloroplasts might be explained as follows. In the cell, the phosphate translocator can accept triose-P and 3-P-glycerate from the cytosol, as well as Pi. In isolated chloroplasts, exchange is with phosphate alone, enhancing the net transport of triose-P from the stroma. If the rate of exchange is too high, exchange of triose-P for Pi can deplete the chloroplast of metabolites and thus inhibit photosynthesis (26).

On the Method. The subcellular fractionation of protoplasts, the details of which have been independently developed by Robinson and Walker (20) and in this laboratory, together with the range of analytical techniques presented in this article provide a powerful system for investigating photosynthesis. This method can also be used with the chromatographic separation of radioactively labeled substances, as will be dealt with in a later publication (Wirtz, Stitt, and Heldt, in preparation). Protoplasts are an experimentally simpler system than whole leaves and can be easily experimentally manipulated. However, although they provide a more complete photosynthetic system, limitations should not be ignored. The extent to which metabolism is altered by the loss of the cell wall and the removal of cellular contacts should be considered. For example, such alterations may be particularly significant in the case of sucrose export. Eventually, analysis of the compartmentation of metabolism in whole leaves may be required. In this context, the early nonaqueous work of Heber and associates (5, 6, 21, 25) is still of importance.

Acknowledgments—The authors are grateful to Prof. M. Klingenberg (Munich) for providing us with the laboratory facilities to carry out this work. We are indebted to Prof. D. Walker, Dr. S. Robinson, and Dr. R. A. Leegood (Sheffield) for their advice in the preparation of protoplasts.

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