Substrate Flow from Photosynthetic Carbon Metabolism to Chloroplast Isoprenoid Synthesis in Spinach
Evidence for a Plastidic Phosphoglycerate Mutase

Detlef Schulze-Siebert, Adolf Heinze, and Gernot Schultz

Botanisches Institut, Tierärztliche Hochschule Hannover, Bünteweg 17d, D-3000 Hannover 71, Bundesrepublik Deutschland

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Dedicated to Professor Helmut Holzer, Freiburg, on the occasion of his 65th birthday

Spinacia oleracea, ß-Carotene Synthesis, Plastoquinone-9 Synthesis, Photosynthetic Carbon Fixation, Phosphoglycerate Mutase in Chloroplasts

The carbon flow from 3-phosphoglycerate to pyruvate and acetyl-CoA within the chloroplast as well as the pathway for the formation of ß-carotene, plastoquinone-9 etc. as plastidic isoprenoids from photosynthetically fixed CO₂ hitherto remained unclear because the presence of the plastidic mutase in chloroplasts had not unequivocally been proven. To clarify this question, the incorporation of 14CO₂ into long chain fatty acids ([12, 13] and literature) was studied using spinach and barley protoplasts and barley seedlings. In protoplasts as well as in seedlings under conditions of CO₂ fixation, the largest portion of the acetyl-CoA derived compounds formed was ß-carotene and plastoquinone-9 rather than fatty acids and sterols. High rates of fatty acid synthesis were obtained by supplying acetate as well. Mevalonate was incorporated into sterols but not into ß-carotene and plastoquinone-9. Direct evidence was obtained for the hitherto questioned plastidic phosphoglycerate mutase. Low activities were found in spinach chloroplasts which were substantiated by the criteria of latency method. Substrate flow from 3-phosphoglycerate to pyruvate via 2-phosphoglycerate and phosphoenolpyruvate was shown by applying [1-14C]-glycerate and following its incorporation. From this the following conclusions were drawn: (i) The plastidic IPP synthesizing system is strongly separated from the cytosolic-ER one. (ii) As the formation of ß-carotene and plastoquinone-9 is favoured under conditions of photosynthetic CO₂ fixation, a direct carbon flow from 3-phosphoglycerate to isoprenoids via acetyl-CoA by a low-capacity pathway, which shows high affinities for the substrates, is suggested.

Abbreviations: DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; IPP, isopentenyldiphosphate; non-reversible and reversible NADP-GPDH, non-reversible and reversible NADP-glyceraldehyde-3-phosphate dehydrogenase; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; 2- and 3-PGA, 2- and 3-phosphoglycerate; PQ, plastoquinone-9; u, unit of enzyme activity, μmol x min⁻¹.

Reprint requests to Prof. Dr. G. Schultz.

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ture cited herein) and aromatic amino acids via plastidic shikimate pathway [14–19] as well as incorporation of phosphoenolpyruvate into aromatic amino acids [20, 21] offer further evidence for a complete pathway from the Calvin cycle to acetyl-CoA in spinach chloroplasts.

Numerous arguments were raised against a plastidic path from 3-PGA to acetyl-CoA for which we have our counter-arguments:

Only low rates of about 0.1 to 1 μmol/mg chlorophyll × h, that is 10⁻³ to 10⁻² of the photosynthetic CO₂-fixation were measured, however, we found that this is fully sufficient for the synthesis of coenzymes etc. in the cell.

Some enzymes are apparently lacking or have been found to be only transient or restricted to distinct developing stages. Indeed, this is a critical point because a co-pathway exists in the cytosol. One should be aware that DHAP and 3-PGA as primary products of photosynthetic CO₂-fixation are effectively shuttled out by the phosphate-transporter against P_i import [22, 23]. Products of glycolysis are formed in the cytosol from exported DHAP and 3-PGA at high rates and serve as substrates for plastidic reactions. For instance, PEP formed in the cytosol is transported back [24] by the phosphate translocator [25, 26]. Pyruvate is imported into the chloroplast at considerable rates at least in Pisum [27] and acetate in spinach [28].

In contrast to oilseed plastids of Ricinus communis [29] and plastids of cauliflower (Brassica oleracea) buds [30, 31], only poor indications were obtained for phosphoglycerate mutase in chloroplasts. Where-as in Pisum this enzyme is absent or indefinitely low in activity [32], in spinach there are some indications for its presence [33]. All other glycolytic enzymes, though at restricted activities, were found in chloroplasts. Those were plastidic isoenzyme of 2-phosphoglycerate hydrolyase, pyruvate kinase [32] and PDC [34, 35] in Pisum; the latter also in spinach [36]. Further unequivocal evidence for a plastidic PDC was presented by studies on the regulation of the pyruvate metabolism in spinach [37, 38]. Under distinct in vitro conditions, the synthesis of branched chain amino acids, alanine, and fatty acids can compete for pyruvate as substrate in isolated intact chloroplasts.

Acetate is imported much more effectively, especially into spinach chloroplasts, than pyruvate [39]. Acetyl-CoA is formed at high rates at the expense of ATP and CoA by the plastidic acetyl-CoA synthetase [40]. Doubtlessly, most of the acetate metabolized in the chloroplast originates from the extraplastidic site [41]. Acetyl-CoA formed by the mitochondrial PDC [42, 43] is cloven by the mitochondrial acetyl-CoA-hydrolase [44, 45] to form acetate which can be transported into the chloroplast for plastidic reactions. In addition acylcarnitine has been detected in plant systems [46, 47], however, it is not proven that it has a similar function as the acyl-transfer system in animal cells.

This paper presents evidence for a chloroplast pathway by which 3-PGA is directly converted to isoprenoids via 2-PGA, PEP, pyruvate and acetyl-CoA. This pathway represents a low-capacity system for the production of acetyl-CoA at rates which are sufficient for supplying isoprenoid synthesis with substrates and apparently is adapted to this synthesis. However, it does not fulfill the demand for substrate supply in fatty acid synthesis, which needs strong support by acetate import from outside the chloroplast.

**Experimental**

**Materials**

[2-¹⁴C]Glycine (1.92 GBq/mmol), [3-¹⁴C]serine (2.04 GBq/mmol), sodium salts of [¹⁴C]bicarbonate (2.00 GBq/mmol), [²⁻¹⁴C]pyruvate (400 MBq/mmol), [⁴⁻¹⁴C]acetate (2.00 GBq/mmol) and N,N,-Dibenzylethlyenediamine-di-D,L-¹⁴C]mevalonate (2.15 GBq/mmol) were obtained from Amersham Buchler, Braunschweig, FRG; D,L-¹⁴C]glyceric acid (2.19 GBq/mmol) was from CEA, Gif-sur-Yvette, France.

The substrates and enzymes for the assay of phosphoglycerate mutase were from Boehringer, Mannheim, FRG.

The TLC plates precoated with silicagel G 1500, LS 254 or with cellulose G 1440, LS 254 were purchased from Schleicher & Schüll, Darmstadt, FRG.

Cellulase TC and pectinase Rohament P5 were from Serva, Heidelberg, FRG, all other biochemicals and solvents were of highest analytical grade and were obtained from Sigma, St. Louis, MO, USA, and E. Merck, Darmstadt, FRG.

**Plant varieties used**

Freshly picked, field-grown spinach (Spinacea oleracea) var. “Butterfly” or greenhouse grown bar-
Intact chloroplasts were isolated either according to the method of Nakatani and Barber [48] (Method A) or of Jensen and Bassham [49] (Method B). Chloroplasts isolated by the former procedure were purified by centrifugation through a linear Percoll (Pharmacia, Uppsala, Sweden) gradient as described previously [21]. Chloroplasts resuspended in isotonic Hepes-Tris medium, pH 7.6; (330 mM sorbitol, 50 mM Hepes, adjusted with Tris to pH 7.6; 0.4 mM MgCl₂) were of 80 to 90% intactness in the ferricyanide test. They were virtually free of contamination from the cytosol (no activities of the non-reversible NADP-GAP DH and the NADP-isocitrate dehydrogenase as marker enzymes of the cytosol were detected) and were only slightly contaminated by peroxisomes (the activity of hydroxyppyruvate reductase as marker enzyme of peroxisomes was about 3% related to chlorophyll contents of the total leaf extract). The media were modified in Method B: for homogenisation of leaves the isotonic Mes-KOH medium, pH 6.5, was used (50 mM Mes, adjusted with KOH to pH 6.5; 330 mM sorbitol; 2 mM MgCl₂; 1 mM MnCl₂; 20 mM NaCl; 0.5 mM KH₂PO₄; 4 mM ascorbate; 4 mM cysteine; 2 mM EDTA) and for resuspending the chloroplast sediment the isotonic Hepes-KOH medium, pH 7.6, was applied (50 mM Hepes, adjusted with KOH to pH 7.6; 330 mM sorbitol; 2 mM MgCl₂; 1 mM MnCl₂; 4 mM ascorbate; 2 mM NaNO₃; 0.5 mM KH₂PO₄; 2 mM EDTA). The last step was repeated twice for purification.

Isolation of spinach and barley protoplasts

Protoplasts were prepared as described in [50]. Protoplasts were purified on a discontinuous gradient and were subsequently used for incubation.

Preparation of chloroplast stroma and cytosol fraction from spinach

The purified intact spinach protoplasts (controlled microscopically) were mechanically ruptured by passing through a 17 µm nylon gauze. Intact chloroplasts were separated from this suspension by centrifugation (threefold for 2 min at 1000 x g). The supernatant was used as cytosol fraction. The chloroplast stroma fraction was obtained by osmotic shock of the chloroplasts in a hypotonic buffer (10 mM Tricine, pH 8; 5 mM MgCl₂; 2 mM DTT). Plastidic membranes were sedimented (175000 x g, 30 min) and discarded.

Reaction mixtures

If not otherwise specified intact chloroplasts using Method A were resuspended in an isotonic Hepes-Tris medium pH 7.6. Chloroplasts according to Method B and enriched fractions of mitochondria and peroxisomes were resuspended in isotonic Hepes-KOH medium pH 7.6. When protoplasts were used, the reaction mixture contained in a final volume of 0.6 ml; 500 mM sorbitol; 5 mM Mes adjusted with KOH to pH 5.5 (in experiments at pH 5.5) or Hepes-Tris pH 7.6 (in experiments at pH 7.6); 1 mM CaCl₂; 0.05% bovine serum albumine; 0.05% polyvinilpyrrolidone; and protoplasts equivalent to 50–100 µg of chlorophyll/ml. The reactions were started by adding the radioactive substrates. The mixtures were kept at 20 ± 2°C in a water bath in the light (10³ W/m², Osram “Argaphot”). Aliquots of 0.2 ml were taken at different times.

Assay of isoprenoids and fatty acids

Barley seedlings were analyzed according to the method described in [51]. If organelle preparations were used, aliquots of 0.2 ml were transferred into 0.75 ml chloroform/methanol (1:2, v/v) and then 0.25 ml chloroform and 0.5 ml water, 100 µg each of β-carotene, PQ, squalene, and 300 µg each of sterols and fatty acids were added as carriers.

The chloroform phase was transferred on silicagel thinlayers for separation of lipids and developed with light petroleum (b.p. 40–60°C)/diethylether (20:1, v/v) (system I). After developing the chromatogram the zones containing PQ (Rᵢ=0.43), squalene (Rᵢ=0.97) and other non cyclic tri- and tetraterpenes like phytoene (detected by quenching under UV) and carotenes (Rᵢ=0.78; visible; in spinach about 90 to 95% β-carotene) were scraped out instantly while still wet to avoid oxidation and eluted three times with 1 ml acetone/methanol (1:1, v/v). The eluates were concentrated under nitrogen in a rotary evaporator to a volume of 0.2 ml. The re-chromatography of PQ (Rᵢ=0.31) was carried out by TLC on cellulose (impregnated with 7% paraffine oil in light
petroleum (b.p. 100–140 °C)) with acetone/methanol (6:1, v/v) (system II) as solvent. The separation of carotene ($R_f$ 0.26) and squalene ($R_f$ 0.59) was achieved on silicagel with n-hexane (system III) as solvent. For determination of radioactivity, the zones were scraped out and counted in 1 ml methanol plus 4 ml Hydroluma (Baker Chemicals, Deventer, The Netherlands) in a scintillation counter (Packard Tricarb 3255).

For detection of sterols and fatty acids, the chromatogram was scanned by a radio scanner (Berthold, Wildbad, FRG) after TLC in system I. The zones in the area of $R_f = 0.0–0.3$ were scraped out and eluted as described above. After evaporation under nitrogen to dryness, the sterols and fatty acids were refluxed in 5 ml methanol/water (1:1, v/v), 0.5 g KOH and a small spatula full of pyrogallol for 60 min. The sterols were transferred to light petroleum (b.p. 40–60 °C) and separated into groups of 4-dimethyl-, 4-monomethylsterols and sterols by TLC on silicagel with light petroleum (b.p. 40–60 °C)/diethylether (1:1, v/v) as solvent. For detection of the sterol groups the chromatograms were sprayed with 20% (w/v) phosphotungsten acid in ethanol and heated to 120 °C.

After the extraction of sterols, the hydrolysate was brought to pH 1–2 with 10 m HCl and fatty acids were extracted with light petroleum (b.p. 40–60 °C) and separated into groups of 4-dimethyl-, 4-monomethylsterols and sterols by TLC on silicagel with light petroleum (b.p. 60–80 °C)/diethylether (1:1, v/v) as solvent (system IV); sitosterol and lanosterol were used as references for the main groups. For detection of the sterol groups the chromatograms were sprayed with 20% (w/v) phosphotungsten acid in ethanol and heated to 120 °C.

Determination of 3- and 2-o-phosphoglycerate, phosphoenolpyruvate and pyruvate formed after administration of [1-14C]o,l-glycerate to chloroplasts

500 μl aliquots of chloroplast suspensions from experiments with labeled glycerate were centrifuged in Eppendorf vessels (Eppendorf Gerätebau, Hamburg, FRG) for 1 min at 1200 × g (Sigma 2 m, Heraeus Christ, Osterode, FRG), the volumes of pellet and supernatant were determined in micro calibrated tubes and the pellet was deproteinized by 1 μl perchlorate for 5 min, neutralized with KOH and re-suspended in 200–300 μl 0.2 m Tris, pH 7.6 at 0 °C. Following centrifugation the aliquots were then divided into 50 μl portions for determining (i) pyruvate, (ii) pyruvate + PEP, (iii) pyruvate + PEP + 2-PGA and (iv) pyruvate + PEP + 2-PGA + 3-PGA. The control value (v) was always subtracted. The intermediates were transformed into lactate by adding the respective enzymes. The 50 μl portions were incubated for 30–40 min at 20 °C in 200 μm Tris, pH 7.6, 8 μm MgSO4, 10 μm KCl, 5.6 μm NADH, 12.7 μm ADP and the following enzymes: for (i) 13.7 u l-lactate dehydrogenase; for (ii) like (i) but additionally 8 μ pyruvate kinase; for (iii) like (ii) but additionally 1.5 μ phosphoglycerate hydrase; for (iv) like (iii) but additionally 5 μ phosphoglycerate mutase + 0.2 μm 2.3 bisphospho-d-glycerate; (v) was without addition. The reaction was terminated by adding 300 μl chloroform/methanol (1:2, v/v; + 100 μg l-lactate as carrier). Following centrifugation for 5 min at 15000 × g (Eppendorf 3200) the upper phase was applied to TLC on silicagel/ghur and 1 μ ammonium acetate pH 5.0/96% ethanol (1:7) as solvent. Lactate ($R_f$ 0.73) was exactly sepa-
rated from glycerate \((R_f 0.3)\) and pyruvate \((R_f 0.87)\) and determined by scintillation counting.

**Measurement of enzyme activities**

The enzyme activities were assayed as described in the following references: Hydroxypyruvate reductase (EC 1.1.1.81) [53]; NADP-isocitrate dehydrogenase (EC 1.1.1.42) [54]; nonreversible NADP-GAP DH (EC 1.2.1.9) [55]; reversible NADP-GAP DH (EC 1.2.1.13) [56]; glycerate kinase (EC 2.7.1.31) [52].

Phosphoglycerate mutase (EC 2.7.5.3) [57] was determined (a) in the forward reaction as follows: for measuring the blank value (caused by interfering reaction of adenylate kinase which generates ATP from ADP and allows 3-PGA oxidation by NADH dependent reaction of endogenous GAP DH) the decrease in absorbance at 340 nm was followed for 5–10 min in a final volume of 3 ml containing 83 mM triethanolaminehydrochloride-NaOH buffer, pH 7.6; 1.6 mM MgSO\(_4\); 0.24 mM NADH; 0.68 mM ADP; 4.7 mM 3-PGA; 10–100 \(\mu\)l supernatant from chloroplasts. The reaction was then followed after adding 8 \(\mu\)l 2-phosphoglycerate hydrolyase, 8 \(\mu\)l pyruvate kinase, 30 \(\mu\)l lactate dehydrogenase, 0.1 mM 2,3-bisphosphoglycerate and equilibrating for at least 5 min. (b) The backward reaction was assayed in a final volume of 1 ml containing: 80 mM triethanolaminehydrochloride-NaOH buffer, pH 7.6; 1.6 mM EDTA; 10 mM MgCl\(_2\); 5 mM glutathione; 5 mM hydrazinesulphate, 15 mM ATP (both neutralized); 0.2 mM NADH; 0.2 mM 2,3-bisphosphoglycerate; 45 \(\mu\)l 3-phosphoglycerate kinase, 8 \(\mu\)l NADP-GAPDH; 10–100 \(\mu\)l chloroplast extract; after an equilibration time of 5 min the reaction was started with 3.2 mM 2-PGA.

In both tests the unspecific oxidation of NADH by chloroplast stroma and the reactions of 3-PGA contaminating 2-PGA (and vice versa) were regarded in the evaluation. The corrected forward reaction was determined to 1.11 nkat/mg chlorophyll (uncorrected 1.30) and the backward reaction was 1.06 nkat/mg chlorophyll. Thus for calculation in Fig. 3 the mean values of both reactions were used.

**Measurements of latency of enzymes**

Latency experiments were carried out as described in [32]. For definition of latency values in \(\%\) see [31].

**Other methods**

\(\text{CO}_2\) fixation rate, chlorophyll as well as protein contents were determined as described in [58].

**Results and Discussion**

**Efficiency of various carbon sources in isoprenoid and fatty acid synthesis of spinach and barley protoplasts and barley seedlings in the light**

Various intermediates of the isoprenoid and fatty acid synthesis were applied to spinach and barley protoplasts and barley seedlings as shown in Table I. A characteristic feature in protoplast studies using NaH\(^{14}\text{CO}_3\) (Table I, Fig. 1 and 2) was that \(\beta\)-carotene and PQ were the only compounds of acetyl-CoA metabolism which were preferentially synthesized from primary products of photosynthesis. Especially at low \(\text{CO}_2\)-supply isoprenoid synthesis was much higher than fatty acid synthesis (Fig. 1). About 0.25 nmol \(\beta\)-carotene and 0.1 nmol PQ per mg chlorophyll \(\times\) h were formed from NaH\(^{14}\text{CO}_3\) (Fig. 1). A similar incorporation pattern but with lower levels were obtained from [2-\(^{14}\text{C}\)]glycine and [3-\(^{14}\text{C}\)]serine indicating a channeling via glycerate (Table I). The poor incorporation of these amino acids was 2.57% for glycine and 4.38% for serine.
Table I. Formation of isoprenoids and fatty acids from different \textsuperscript{14}C-labeled precursors by protoplasts from spinach and barley in reaction media of different pH and by excised barley seedlings. Isolated protoplasts equivalent to 50-100 ng of chlorophyll/ml were resuspended in 500 mM sorbitol; 5 mM Mes, adjusted with KOH to pH 5.5 or with Hepes-Tris to pH 7.6; 1 mM CaCl\textsubscript{2}; 0.05% bovine serum albumine; 0.05% polyvinylpyrrolidone. The rates of photosynthetic CO\textsubscript{2} fixation were between 73 to 79 \textmu mol/mg chlorophyll \times h. The reaction was started with 0.5 mM NaH\textsubscript{14}CO\textsubscript{3}, or with one of the following labeled compounds at 0.1 mM: [2-\textsuperscript{14}C]glycine, [3-\textsuperscript{14}C]serine, [2-\textsuperscript{14}C]pyruvate, [2-\textsuperscript{14}C]acetate and [2-\textsuperscript{14}C]mevalonate, respectively. The final volume was 0.6 ml. Aliquots were taken after 60 min. When barley seedlings were used 30 excised seedlings were incubated with one of the labeled compounds and illuminated with Philips MRL 160 lamps for 60 min. The reactions were terminated as described in "Experimental".

In contrast to the experiments employing NaH\textsubscript{14}CO\textsubscript{3}, high amounts of fatty acids and sterols

<table>
<thead>
<tr>
<th>Species and pH of reaction medium</th>
<th>Substrates</th>
<th>Sum of lipids (nmol acetate units formed/mg chlorophyll \times h)</th>
<th>Non-cyclical terpenes (squalene, phytol, etc.)</th>
<th>Sterols</th>
<th>( \beta )-Carotene</th>
<th>PQ</th>
<th>Fatty acids</th>
<th>Ratio of formation of ( \beta )-carotene + PQ to sterols</th>
<th>Ratio of formation of ( \beta )-carotene + PQ to fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach protoplasts, pH 7.6</td>
<td>NaH\textsuperscript{14}CO\textsubscript{3}</td>
<td>5.1 ± 0.3 (6)</td>
<td>0.51</td>
<td>0.67</td>
<td>1.18</td>
<td>1.12</td>
<td>1.58</td>
<td>3.43</td>
<td>1.46</td>
</tr>
<tr>
<td>Spinach protoplasts, pH 5.5</td>
<td>[2-\textsuperscript{14}C]glycine</td>
<td>0.3 ± 0.1 (3)</td>
<td>0.07</td>
<td>0.04</td>
<td>0.03</td>
<td>0.06</td>
<td>0.10</td>
<td>2.25</td>
<td>0.90</td>
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<tr>
<td>Spinach protoplasts, pH 5.5</td>
<td>[3-\textsuperscript{14}C]serine</td>
<td>1.2 ± 0.1 (3)</td>
<td>0.17</td>
<td>0.22</td>
<td>0.17</td>
<td>0.29</td>
<td>0.36</td>
<td>2.09</td>
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<tr>
<td>Spinach protoplasts, pH 5.5</td>
<td>[2-\textsuperscript{14}C]pyruvate</td>
<td>10.3 ± 3.0 (3)</td>
<td>0.52</td>
<td>0.31</td>
<td>0.11</td>
<td>0.11</td>
<td>9.27</td>
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<tr>
<td>Spinach protoplasts, pH 5.5</td>
<td>[2-\textsuperscript{14}C]acetate</td>
<td>33.3 ± 6.0 (4)</td>
<td>1.67</td>
<td>2.00</td>
<td>0.33</td>
<td>0.67</td>
<td>28.64</td>
<td>0.50</td>
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<tr>
<td>Spinach protoplasts, pH 5.5</td>
<td>[2-\textsuperscript{14}C]mevalonate</td>
<td>3.5 ± 0.6 (2)</td>
<td>1.65</td>
<td>1.80</td>
<td>0.03</td>
<td>0.03</td>
<td>#</td>
<td>0.03</td>
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<tr>
<td>Excised barley seedlings, pH 7.6</td>
<td>NaH\textsuperscript{14}CO\textsubscript{3}</td>
<td>6.9 ± 0.5 (4)</td>
<td>0.69</td>
<td>1.20</td>
<td>1.40</td>
<td>1.31</td>
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<tr>
<td>Excised barley seedlings, pH 5.5</td>
<td>[2-\textsuperscript{14}C]glucose</td>
<td>1.4 ± 0.2 (3)</td>
<td>0.31</td>
<td>0.26</td>
<td>0.06</td>
<td>0.28</td>
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<td>Excised barley seedlings, pH 5.5</td>
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<td>3.1 ± 0.1 (3)</td>
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<td>Excised barley seedlings, pH 5.5</td>
<td>[2-\textsuperscript{14}C]acetate</td>
<td>39.8 ± 10.0 (4)</td>
<td>0.88</td>
<td>1.20</td>
<td>0.08</td>
<td>1.03</td>
<td>36.60</td>
<td>0.93</td>
<td>0.03</td>
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<tr>
<td>Excised barley seedlings, pH 5.5</td>
<td>[2-\textsuperscript{14}C]mevalonate</td>
<td>5.0 ± 0.8 (2)</td>
<td>1.04</td>
<td>3.87</td>
<td>0.03</td>
<td>0.06</td>
<td>#</td>
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</tbody>
</table>

* The calculations are based on the following assumptions: a) 1 mol NaH\textsuperscript{14}CO\textsubscript{3} is equivalent to 0.5 mol labeled acetate formed (1 labeled acetate formed corresponds to 1 acetate unit); b) 1 mol [2-\textsuperscript{14}C]glycine is equivalent to 0.5 mol acetate units (if [2-\textsuperscript{14}C]glycine is metabolized via glycolate pathway, then C\textsubscript{2} and C\textsubscript{3}-atom of the serine formed is labeled); c) 1 mol [3-\textsuperscript{14}C]-serine, [2-\textsuperscript{14}C]pyruvate and [2-\textsuperscript{14}C]acetate, respectively, is equivalent to 1 mol acetate units formed; d) 1 mol [2-\textsuperscript{14}C]mevalonate is equivalent to 3 mol acetate units formed.

\# Only insignificant amounts of fatty acids were formed.

acids in protoplasts may be caused by low rates of transport through the plasmalemma membrane [59, 60]. It is noteworthy that the synthesis of sterols in the cytosol is more or less discriminated against the ones of \( \beta \)-carotene and PQ in chloroplasts [1] (average ratio of \( \beta \)-carotene + PQ / sterols in Table I was 2.4). Moreover in agreement to [28, 39] fatty acids were formed only at low rates under these conditions (average ratio of \( \beta \)-carotene + PQ / fatty acids in Table I was 1.1). The rates of synthesis of \( \beta \)-carotene from NaH\textsuperscript{14}CO\textsubscript{3} in protoplasts may reflect the situation under steady state conditions of differentiated cells. The rates of seedlings were not higher (Table I).

In contrast to the experiments employing NaH\textsuperscript{14}CO\textsubscript{3}, high amounts of fatty acids and sterols
Fig. 2. Incorporation of $^{14}$C-labeled precursors into lipids by isolated spinach protoplasts in the light. The reaction was started with 0.5 mM NaH$^{14}$CO$_3$ or with one of the following compounds at 0.1 mM: [2-$^{14}$C]pyruvate, [2-$^{14}$C]acetate and [2-$^{14}$C]mevalonate. For assay conditions and definitions of acetate units see Table I. Each value was averaged from at least 2 experiments. Å, fatty acids; ▲, non-cyclic di- and triterpenes; ♦, sterols; ○, β-carotene; □, PQ. Following values are to be multiplied: by 16 Å$^*$; by 10 ▲$^*$, by 6 ♦$^*$.

were formed when [2-$^{14}$C]acetate were added. The amounts of β-carotene and PQ were almost equal to that in experiments with NaH$^{14}$CO$_3$. When [2-$^{14}$C]pyruvate was provided as a carbon source only low amounts but the same incorporation pattern as with acetate was observed. A slow non-enzymatic conversion of pyruvate to acetate during incubation is possible so that incorporation from [2-$^{14}$C]pyruvate may be attributable to acetate formed. The low average ratio of β-carotene + PQ / fatty acids in Table I of 0.03 may be explained by saturation of plastidic isoprenoid synthesis under these conditions since the absolute values resembled the maximal values obtained by applying NaH$^{14}$CO$_3$. Lowering the pH from 7.6 to 5.5 in the suspension medium to increase the portion of undissociated acid only increased the incorporation rates of acetate only into fatty acids by a factor 5.

In protoplasts and in seedlings [1] [2-$^{14}$C]mevalonate was only incorporated into sterols formed outside the chloroplast. In barley after 3 h incubation, predominantly 4-dimethylsterols (about 75%) and also 4-methylsterols (15%) were formed but only some sterols (about 5%). The sterol groups were routinely analyzed together and named as sterols in Table I and in Fig. 1 and 3. The fractions of β-carotene and PQ depicted in Table I and Fig. 2 were virtually free of radioactivity.

In the time course in Fig. 1 it is shown that spinach protoplasts formed isoprenoids and fatty acids from all the added intermediates of the photosynthetic carbon metabolism at largely constant rates over a period of 60 min in the light. Even if NaH$^{14}$CO$_3$ was supplied, a constant rate was obtained even though CO$_2$ was consumed within 4 min. As results in Fig. 1 indicate, the pool of primary products of photosynthesis formed may suffice for the synthesis of isoprenoids and amino acids in chloroplasts over a prolonged period. Only 0.01% of the primary products were consumed in these syntheses.

The results in Table I convincingly shows that the largest portion of the acetyl-CoA derived compounds, known to be formed in chloroplasts under conditions of photosynthetic CO$_2$ fixation, was β-carotene plus PQ but not fatty acids. The high rates of fatty acid synthesis observed elsewhere were only obtained when acetate was used as substrate. Acetate also strongly enhanced sterol synthesis in the cytosol. This finding together with the fact (Table I, see also [1, 4]) that mevalonate only favours sterol synthesis in the cytosol and ER but not of plastidic isoprenoids indicates that the plastidic isoprenoid synthesis is directly supplied by the photosynthetic carbon metabolism. To obtain evidence for such a pathway, it was necessary to re-examine whether a phosphoglycerate mutase is indeed present in spinach chloroplasts. This was undertaken in the following two ways.

Identification of phosphoglycerate mutase in spinach chloroplasts

To prove the existence of plastidic phosphoglycerate mutase, highly purified spinach chloroplasts were
used (see Experimental). From these the enzymes of chloroplast stroma were set free according to the method of successive osmotic lysis as described in [32], chloroplasts were centrifuged and the supernatant was assayed for enzyme activity. Reversible NADP-GAPDH and shikimate oxidoreductase [14] were used as marker enzymes for chloroplast stroma in spinach. As shown in Fig. 3, identical behaviour during lysis can be shown by comparing activities of the phosphoglycerate mutase studied and the two marker enzymes. The activity of the mutase in the supernatant reached values of maximally 8 but on the average 1 nkat/mg chlorophyll and resembled other enzymes of anabolic pathways in chloroplasts. Compared to the total activity in the cell homogenate of the mutase of 590 nkat/mg chlorophyll the plastidic activity was 1.4%.

To evaluate a possible loose absorption of cytosolic activities at the outer chloroplast envelope membrane, the phosphoglycerate mutase activity in the supernatant of fully intact chloroplasts was determined for phosphoglycerate mutase activity after washing with and without 100 mM KCl in isotonic Hepes-Tris medium, pH 7.6. The value after washing with KCl was 0.126; after washing without KCl 0.133 nkat/mg chlorophyll. The sorbitol concentration of the osmoticum did not significantly influence the activity of the mutase; the activity at 10 mM sorbitol was 1.60 and after adjustment to 330 mM it was 1.67 nkat/mg chlorophyll. The plastidic enzyme has a pH optimum between 8.2 and 8.6 in triethanolamine buffer differing from the enzyme from rabbit muscle which has a pH optimum of 5.9 [61].

Formation of 3- and 2-phosphoglycerate, phosphoenolpyruvate and pyruvate in chloroplasts from added [1-14C]D,L-glycerate

Glycerate kinase forms PGA from D-glycerate which arises from photorespiratory carbon pathway in leaf peroxisomes and mitochondria. By determining glycerate kinase activities in chloroplasts and the extraplastic space (“cytosol”) the findings of Schmitt and Edwards [9] were confirmed that the kinase solely occurs in the chloroplast stroma. Reversible NADP-GAPDH and shikimate oxidoreductase were used as marker enzymes for the chloroplast stroma and non-reversible NADP-GAPDH and NADP-isocitrate dehydrogenase for the cytosol (data not shown). It could be shown by chromatographic analysis of the products formed that the chloroplast enzyme solely formed 3-PGA; this agrees with results in ref. [10]. The chromatographic analysis was verified by transforming the isolated 3-PGA to 2-PGA by addition of rabbit phosphoglycerate mutase.

Since it is established that glycerate kinase is localized in chloroplasts it was used as a tool to identify phosphoglycerate mutase in this organelle. Since the mutase was suggested to participate in the reaction sequence in chloroplasts: Glycerate $\rightarrow$ 3-PGA $\rightarrow$ 2-PGA $\rightarrow$ PEP $\rightarrow$ Pyruvate, [1-14C]D,L-glycerate was added to study this reaction sequence. Following addition of labeled substrate and illumination for 60 min, chloroplasts were sedimented by centrifugation at 1,500 x g for 1 min, the chloroplast pellet and...
the supernatant medium were then checked for the labeled intermediates. Because of the low concentration of the labeled intermediates formed, the enzyme tests for converting all intermediates into lactate were combined with radiochromatography (see Experimental).

To calculate the concentration of intermediates within the chloroplast, that is the sorbitol-impermeable H₂O space of chloroplasts [23] confined by the inner envelope membrane in which the considered reactions occur, the following assumptions were made. The chloroplast pellet consists of the sorbitol-impermeable H₂O space of chloroplasts and the external space, that is the interorganelle space and the permeable intermembranous space between inner and outer envelope membranes. The sorbitol-impermeable space had a volume of 38 µl/mg chlorophyll at a sorbitol concentration of 330 mM as osmoticum as determined by Heber and Heldt [23]. The values for external space after centrifugation at 1,500 x g for 5 min, ascertained by these means, was 40 µl chlorophyll. If it is assumed that the external space has the same concentration as the supernatant medium, the chloroplast values for PEP and 2-PGA but also for pyruvate were much too high and seemed to be implausible because the equilibrium constant for the phosphoglycerate mutase reaction K₃-PGA/2-PGA is about 5 [61]. To obtain more correct values for chloroplast impermeable space from the analytical data of the chloroplast pellet, we developed a conclusive working hypothesis [21] which was applied here as well (Fig. 4). It was assumed that the above intermediates were more or less equally distributed within the chloroplast pellet during centrifugation procedure of the long time light treated chloroplasts, which had became fragile and loose intermediates.

Though the test system was of restricted accuracy remarkable differences were obtained between the concentration in chloroplasts and in supernatant medium (Fig. 4). Only chloroplasts contained the intermediates of interest, 3- and 2-PGA, PEP and pyruvate, at higher concentrations so that it can be claimed that phosphoglycerate mutase reaction occurs in the chloroplast as well. The supernatant medium contained only 3-PGA at higher concentrations attributable to exchange by the phosphate translocator. The low concentration of 2-PGA, PEP and pyruvate found in the supernatant medium indicated a negligible contamination by cytosolic phosphoglycerate mutase, a low transfer rate of these intermediates particularly 2-PGA across the chloroplast envelope membranes and a high intactness of chloroplasts.

Concluding Remarks

The last link between 3-PGA and acetyl-CoA, phosphoglycerate mutase, was identified to be in the stroma of spinach chloroplasts. This means that all enzymes, 2-phosphoglycerate hydrolyase, pyruvate kinase [32, 33] as well as PDC [34–36], are present in the chloroplast enabling a purely plastidic flow from 3-PGA to acetyl-CoA. This flow, at least in spinach, may be understood as a low capacity but highly effective flow for supplying essential pathways such as the plastidic isoprenoid synthesis. This is confirmed in another study as well ([62, 63] and D. Schulze-Siebert and G. Schultz, in preparation). The glycolytic metabolism active in the cytosol is linked to the chloroplast photosynthetic carbon fixation by
Fig. 5. Proposed scheme of carbon flow from primary photosynthetic products to isoprenoid compounds, fatty acids and other compounds (amino acids, etc.) in spinach chloroplasts. Based on results of this paper and in context with earlier studies [23], the scheme should focus on the following points: (i) A carbon flow from 3-PGA to 2-PGA, PEP and pyruvate within the chloroplast (and to some extent outside the chloroplast [26]) exists to provide substrates for the synthesis of plastidic isoprenoid compounds (PQ, α-tocopherol, β-carotene, etc.) and other compounds (branched-chain amino acids Val, Leu and Ile, aromatic amino acids Phe, Tyr and Trp and other amino acids, etc.). (ii) Plastidic PDC and plastidic isoprenoid synthesis are more or less channeled systems. (iii) The main carbon source for the fatty acid synthesis in spinach chloroplasts is acetate imported from the cytosol. E’ase, 2-phosphoglycerate hydrolyase, enolase; GK, glycerate kinase; MITO, mitochondrion; PER, peroxysome; PGM, phosphoglycerate mutase; PK, pyruvate kinase; Pyr, pyruvate; αT, α-tocopherol.

the phosphate translocator shuttle which exchanges DHAP but also 3-PGA and less amounts of PEP between the two compartments [24]. There are good reasons that both pathways may mutually compete under certain conditions. Furthermore, acetate in spinach, but pyruvate in Pisum [27] is transferred by mediated diffusion across the envelope membranes into chloroplasts.

Based on the results from isolated enzymes it was concluded [36] that in chloroplasts the formation of acetyl-CoA from pyruvate by the plastidic PDC competes with that from acetate by the plastidic acetyl-CoA synthetase. However, from present results it can be inferred that under photosynthetic conditions acetyl-CoA synthesis by plastidic PDC favours the formation of plastidic isoprenoids, β-carotene and PQ, whereas acetyl-CoA formed from imported acetate only dramatically enhances the fatty acid synthesis. Formation of β-carotene and PQ favoured under photosynthetic conditions (Table I, Fig. 1 and 2) indicate a metabolic channeling of the PDC and IPP synthesizing system which exists apart from the plastidic fatty acid synthetase. Nevertheless, the metabolic channeling of the PDC-IPP synthesizing system is only a relative one. The plastidic PDC is not only involved in the isoprenoid synthesis but is additionally engaged in the synthesis of branched chain amino acids [21, 37, 38]. Under certain conditions, probably artificial ones, the plastidic IPP synthesizing system [64] can also be supported from extraplasmatic acetate. The flow diagram in Fig. 5 illustrates the situation in chloroplasts as based on the results for spinach.

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