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

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

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The carbon flow from 3-phosphoglycerate to pyruvate and acetyl-CoA within the chloroplast as well as the pathway for the formation of β-carotene, plastoquinone-9 etc. as plastidic isoprenoids from photosynthetically fixed CO_2 hitherto remained unclear because the presence of the phosphoglycerate mutase in chloroplasts had not unequivocally been proven. To clarify this question, the incorporation of $^{14}CO_2$, [2-^{14}C]acetate, [2-^{14}C]pyruvate, [2-^{14}C]glycine, [3-^{14}C]serine and [2-^{14}C]mevalonate into β-carotene, plastoquinone-9, sterols and fatty acids was studied using spinach and barley protoplasts and barley seedlings. In protoplasts as well as in seedlings under conditions of CO_2 fixation, the largest portion of the acetyl-CoA derived compounds formed was β-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 β-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-^{14}C]-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 β-carotene and plastoquinone-9 is favoured under conditions of photosynthetic CO_2 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.

Introduction

Goodwin [1] published evidence that a complete pathway from photosynthetic CO_2-fixation to the synthesis of acetyl-CoA and IPP exists in chloroplasts. Comparing the incorporation of $^{14}CO_2$ and [2-^{14}C]mevalonate into isoprenoids of maize seedlings, barley, oats, lettuce and peas, indicated a plastidic and independently an extraplastidic IPP-synthesis *via* mevalonate. Recently, plastidic and ER-cytosolic isoenzymes of HMG-CoA-reductase and mevalonate kinase were identified in *Nepeta* and spinach [2] and of HMG-CoA-reductase in *Pisum* [3]. The hypothesis for a complete $CO_2 \rightarrow$ acetyl-CoA substrate flow was supported by results of applying [1- and 2-^{14}C]glycine and [U-^{14}C]serine, both are well known as intermediates in photorespiratory carbon pathway, which were incorporated into plastidic isoprenoids [4, 5]. Only β-carotene and the phytyl moiety of chlorophyll were labeled, while the sterols were scarcely labeled. It was concluded that acetyl-CoA could be formed from 3-PGA of the Calvin-cycle but could also be derived from D-glycerate of the photorespiratory carbon pathway. D-Glycerate is transported by the D-glycerate/glycolate carrier [6–8] from peroxisomes into chloroplasts and returned to the PGA-metabolism by the exclusively plastidic enzyme, glycerate kinase [9], forming only 3-PGA from glycerate [10] and never 2-PGA as compared to the liver enzyme [11]. Incorporation of $^{14}CO_2$ into long chain fatty acids ([12, 13] and litera-

Abbreviations: DHAP, dihydroxyacetonephosphate; DTT, dithiothreitol; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; IPP, isopentenyldiphosphate; non-reversible and reversible NADP-GPDH, non-reversible and reversible NADP$^+$-D-glyceraldehyde-3-phosphate dehydrogenase; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; 2- and 3-PGA, 2-D- and 3-D-phosphoglycerate; PQ, plastoquinone-9; u, unit of enzyme activity, $\mu mol \times min^{-1}$.

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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 CO_2-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 also 3-PGA as primary products of photosynthetic CO_2-fixation are effectively shuttled out by the phosphate-translocator 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. Whereas 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 chloroplast 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 acyltransfer 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-^{14}C]Glycine (1.92 GBq/mmol), L-[3-^{14}C]serine (2.04 GBq/mmol), sodium salts of [^{14}C]bicarbonate (2.00 GBq/mmol), [2-^{14}C]pyruvate (400 MBq/mmol), [2-^{14}C]acetate (2.00 GBq/mmol) and N,N,-Dibenzylethylenediamine-di-D,L-[2-^{14}C]mevalonate (2.15 GBq/mmol) were obtained from Amersham Buchler, Braunschweig, FRG; D,L-[1-^{14}C]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, Einbeck, 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 *(Spinacia oleracea)* var. "Butterfly" or greenhouse grown bar-

ley *(Hordeum vulgare)* var. "Aramir" were used for all experiments. The caryopses (grains) of barley were germinated on filter paper in the light (daylight or Philips MRL 160, 430 W/m^3, distance 0.3 m).

Isolation of spinach chloroplasts

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 $MgCl_2$) were of 80 to 90% intactness in the ferricyanide test. They were virtually free of contaminations 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 hydroxypyruvate 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 $MgCl_2$; 1 mM $MnCl_2$; 20 mM NaCl; 0.5 mM KH_2PO_4; 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 $MgCl_2$; 1 mM $MnCl_2$; 4 mM ascorbate; 2 mM $NaNO_3$; 0.5 mM KH_2PO_4; 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 $1000 \times g$). 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 $MgCl_2$; 2 mM DTT). Plastidic membranes were sedimented ($175000 \times g$, 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 $CaCl_2$; 0.05% bovine serum albumine; 0.05% polyvinylpyrrolidone; 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 (10^3 W/m^2, 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 ($R_f = 0.43$), squalene ($R_f = 0.97$) and other non cyclic tri- and tetraterpenes like phytoene (detected by quenching under UV) and carotenes ($R_f = 0.78$; 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 (R_f 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. 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.

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). The acids were evaporated under nitrogen to dryness and estered in 2.5 ml methanol/benzene/H_2SO_4 solution (100:5:5, v/v/v) at 80 °C for 60 min in stoppered vials. The methylesters of fatty acids were then extracted with light petroleum and separated into groups of methyl esters of saturated (14:0 + 16:0 + 18:0), mono- (16: + 18:1), di- (16:2 + 18:2) and triunsaturated (16:3 + 18:3) fatty acids by TLC on silicagel (impregnated with 5% $AgNO_3$ in acetonitrile) with *n*-hexane/diethylether (85:15, v/v) as solvent (system V). The esters were visualized under UV 366 nm after spraying with 0.2% (w/v) 2,7-dichlorofluorescein in ethanol. In Tables and Figures the values of the four groups of fatty acids were counted together.

Separation of 3-D-phosphoglycerate and 2-D-phosphoglycerate

200 µg each of 3-PGA and 2-PGA were added as carrier into the organelle reaction mixture terminated as described in [52]. TLC of products was performed on cellulose with methanol/NH_3/water (6:1:3, v/v/v) (system VI). The R_f values were as follows: glycerate 0.81; 3-PGA 0.56; 2-PGA 0.47. For detection, the references and samples were treated with 0.15% (w/v) $FeCl_3 \times 6\ H_2O$ in acetone/0.3 M HCl (97:3, v/v) and after heating to 120 °C the products were visualized by spraying with 1.2% (w/v) sulphosalicylic acid in acetone. In additional experiments, the isolated 3-PGA was transformed to 2-PGA by adding phosphoglycerate mutase from rabbit muscle and re-analyzed by TLC. The detected zones were scraped out for determination of radioactivity.

Determination of 3- and 2-D-phosphoglycerate, phosphoenolpyruvate and pyruvate formed after administration of [1-^{14}C]D,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 M 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 mM Tris, pH 7.6, 8 mM $MgSO_4$, 10 mM KCl, 5.6 mM NADH, 12.7 mM ADP and the following enzymes: for (i) 13.7 u L-lactate dehydrogenase; for (ii) like (i) but additionally 8 u pyruvate kinase; for (iii) like (ii) but additionally 1.5 u phosphoglycerate hydrolyase; for (iv) like (iii) but additionally 5 u phosphoglycerate mutase + 0.2 mM 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 M 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 µl supernatant from chloroplasts. The reaction was then followed after adding 8 u 2-phosphoglycerate hydrolyase, 8 u pyruvate kinase, 30 u 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 u 3-phosphoglycerate kinase, 8 u NADP-GAPDH; 10–100 µl 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

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 $NaH^{14}CO_3$ (Table I, Fig. 1 and 2) was that β-carotene and PQ were the only compounds of acetyl-CoA metabolism which were preferentially synthesized from primary products of photosynthesis. Especially at low CO_2-supply isoprenoid synthesis was much higher than fatty acid synthesis (Fig. 1). About 0.25 nmol β-carotene and 0.1 nmol PQ per mg chlorophyll × h were formed from $NaH^{14}CO_3$ (Fig. 1). A similar incorporation pattern but with lower levels were obtained from [2-^{14}C]glycine and [3-^{14}C]serine indicating a channeling via glycerate (Table I). The poor incorporation of these amino

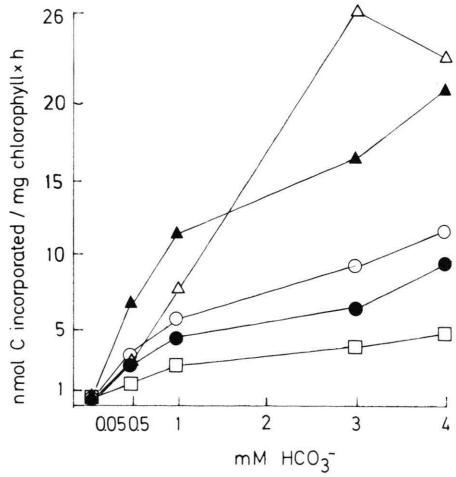

Fig. 1. Formation of PQ, β-carotene, sterols and non-cyclisized tri- and tetraterpenes by protoplasts in dependence of added $NaH^{14}CO_3$ concentration. The chlorophyll contents were 0.1 mg/ml protoplast suspension, the rate of CO_2 fixation was 79 µmol/mg chlorophyll × h and the illumination time was 60 min. For further details see Experimental. △, fatty acids; ▲, non-cyclic tri- and tetraterpenes; ○, β-carotene; ●, sterols; □, PQ.

Table I. Formation of isoprenoids and fatty acids from different ^{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 µg 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 $CaCl_2$; 0.05% bovine serum albumine; 0.05% polyvinylpyrrolidine. The rates of photosynthetic CO_2 fixation were between 73 to 79 µmol/mg chlorophyll × h. The reaction was started with 0.5 mM $NaH^{14}CO_3$ or with one of the following labeled compounds at 0.1 mM: [2-^{14}C]glycine, [3-^{14}C]serine, [2-^{14}C]pyruvate, [2-^{14}C]acetate and [2-^{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".

Species and pH of reaction medium	Substrates	Sum of lipids analyzed	Non-cyclical terpenes (squalene, phytoene, etc.)	Sterols	β-Carotene	PQ	Fatty acids	Ratio of formation of β-carotene + PQ to sterols	Ratio of formation of β-carotene + PQ to fatty acids
		nmol acetate units formed/mg chlorophyll × h*							
Spinach protoplasts pH 7.6	$NaH^{14}CO_3$	5.1 ± 0.3 (6)	0.51	0.67	1.18	1.12	1.58	3.43	1.46
	[2-^{14}C]glycine	0.3 ± 0.1 (3)	0.07	0.04	0.03	0.06	0.10	2.25	0.90
	[3-^{14}C]serine	1.2 ± 0.1 (3)	0.17	0.22	0.17	0.29	0.36	2.09	1.27
	[2-^{14}C]pyruvate	10.3 ± 3.0 (3)	0.52	0.31	0.11	0.11	9.27	0.71	0.02
	[2-^{14}C]acetate	33.3 ± 6.0 (4)	1.67	2.00	0.33	0.67	28.64	0.50	0.03
	[2-^{14}C]mevalonate	3.5 ± 0.6 (2)	1.65	1.80	0.03	0.03	#	0.03	—
Spinach protoplasts pH 5.5	[2-^{14}C]glycine	0.9 ± 0.2 (3)	0.27	0.11	0.12	0.14	0.23	2.36	1.13
	[3-^{14}C]serine	1.3 ± 0.2 (3)	0.42	0.12	0.20	0.21	0.36	3.42	1.14
	[2-^{14}C]pyruvate	10.7 ± 4.0 (3)	0.64	0.75	0.32	0.32	8.67	0.85	0.07
	[2-^{14}C]acetate	184.0 ± 23.0 (4)	3.69	5.54	0.93	0.93	173.60	0.34	0.01
	[2-^{14}C]mevalonate	4.2 ± 1.2 (2)	2.01	2.07	0.02	0.06	#	0.04	—
Barley protoplasts pH 7.6	$NaH^{14}CO_3$	6.9 ± 0.5 (4)	0.69	1.20	1.40	1.31	2.35	2.26	1.15
Barley protoplasts pH 5.5	[2-^{14}C]glycine	1.4 ± 0.2 (3)	0.31	0.26	0.06	0.28	0.45	1.31	0.76
	[3-^{14}C]serine	3.1 ± 0.1 (3)	0.78	0.65	0.12	0.56	0.99	1.05	0.69
	[2-^{14}C]pyruvate	18.4 ± 7.0 (3)	0.37	0.52	0.04	0.37	17.10	0.79	0.02
	[2-^{14}C]acetate	39.8 ± 10.0 (4)	0.88	1.20	0.08	1.03	36.60	0.93	0.03
	[2-^{14}C]mevalonate	5.0 ± 0.8 (2)	1.04	3.87	0.03	0.06	#	0.02	—
Excised barley seedlings	$NaH^{14}CO_3$	3.8 ± 0.3 (3)	n.d.	2.28	0.53	0.13	0.86	0.29	0.77
	$^{14}CO_2$	6.1	n.d.	2.42	0.97	0.25	2.42	0.50	0.50
	[2-^{14}C]glycine	5.1 ± 0.6 (2)	n.d.	2.86	0.29	0.29	1.74	0.20	0.33
	[3-^{14}C]serine	2.2 ± 0.2 (2)	n.d.	0.75	0.50	0.11	0.84	0.81	0.73
	[2-^{14}C]pyruvate	26.5 ± 3.0 (2)	n.d.	21.20	0.53	0.53	4.24	0.05	0.25
	[2-^{14}C]acetate	30.3 ± 4.0 (2)	n.d.	22.10	0.30	0.91	7.00	0.05	0.17
	[2-^{14}C]mevalonate	56.1	n.d.	56.07	0.006	0.03	#	0.0006	—

* The calculations are based on the following assumptions: a) 1 mol $NaH^{14}CO_3$ is equivalent to 0.5 mol labeled acetate formed (1 labeled acetate formed corresponds to 1 acetate unit); b) 1 mol [2-^{14}C]glycine is equivalent to 0.5 mol acetate units (if [2-^{14}C]glycine is metabolized *via* glycolate pathway, then C_2- and C_3-atome of the serine formed is labeled); c) 1 mol [3-^{14}C]-serine, [2-^{14}C]pyruvate and [2-^{14}C]acetate, respectively, is equivalent to 1 mol acetate units formed; d) 1 mol [2-^{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 plasmalemna membrane [59, 60]. It is noteworthy that the synthesis of sterols in the cytosol is more or less discriminated against the ones of β-carotene and PQ in chloroplasts [1] (average ratio of β-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 β-carotene + PQ / fatty acids in Table I was 1.1). The rates of synthesis of β-carotene from $NaH^{14}CO_3$ 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 $NaH^{14}CO_3$, high amounts of fatty acids and sterols

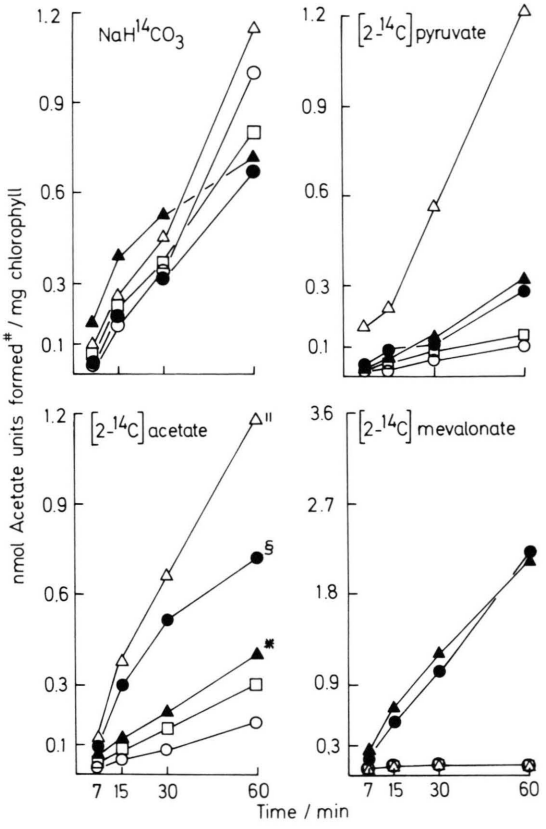

Fig. 2. Incorporation of ^{14}C-labeled precursors into lipids by isolated spinach protoplasts in the light. The reaction was started with 0.5 mM $NaH^{14}CO_3$ or with one of the following compounds at 0.1 mM: [2-^{14}C]pyruvate, [2-^{14}C]acetate and [2-^{14}C]mevalonate. For assay conditions and # for definations of acetate units see Table I. Each value was averaged from at least 2 experiments. △, fatty acids; ▲, non-cyclic di- and triterpenes; ●, sterols; ○, β-carotene; □, PQ. Following values are to be multiplied: by 16 △″; 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%.

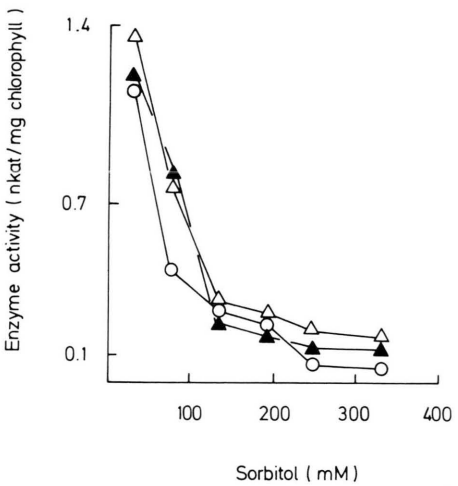

Fig. 3. Identification of phosphoglycerate mutase in intact purified spinach chloroplasts by measuring the latency of the enzyme. As described in Experimental, aliquots of purified spinach chloroplasts isolated according to Method A were incubated 5 min at 25 °C in media of varying molarity of sorbitol as indicated in the figure to ensure lysis of chloroplasts (for defination of latency see [32]). The more the molarity was decreased, the more effective the lysis of chloroplasts was. Following centrifugation at 1,500 × *g* for 1 min, the activity of reversible NADP-GAPDH (○, the value of the ordinate is to be multiplied by 100), shikimate oxidoreductase (△) and phosphoglycerate mutase (▲) originating from the portion of chloroplasts lysed under the respective conditions were measured in the supernatant. The latency values in % [31] / that is (activity after lysis, here the value in 30 mM sorbitol, minus the activity before lysis, here in 330 mM sorbitol, divided by the activity after lysis) × 100 / were: for reversible NADP-GAPDH 95%, for shikimate oxidoreductase 87% and for phosphoglycerate mutase 89%. The rate of photosynthetic CO_2 fixation of non-lysed intact chloroplasts was 73 μmol CO_2/mg chlorophyll × h.

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-^{14}C]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 → 3-PGA → 2-PGA → PEP → Pyruvate, [1-^{14}C]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 × *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 H_2O 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 H_2O 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 $1{,}500 \times g$ 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 $K_{3\text{-PGA/2-PGA}}$ 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.

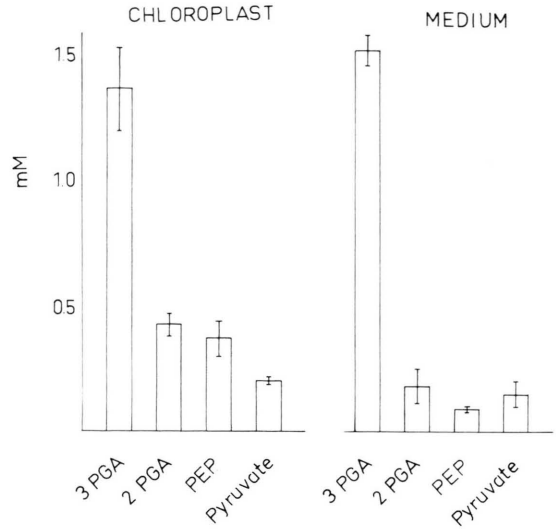

Fig. 4. Distribution between chloroplasts and medium of labeled 3-PGA, 2-PGA, PEP, and pyruvate formed from [1-^{14}C]D,L-glycerate by isolated, intact chloroplasts in the light. Chloroplasts were isolated according to Method B. Following addition of labeled glycerate at final concentration of 5 mM, the chloroplast suspension containing 1.0 mg chlorophyll/ml was illuminated for 60 min. The data represents the average values of three independent experiments; the bars indicate the mean deviation. For determination of labeled products in chloroplast and medium see Experimental. For calculation of the concentration in chloroplasts impermeable space (named chloroplasts in the figure) see text, the supernatant medium after centrifugation is named medium in the figure.

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

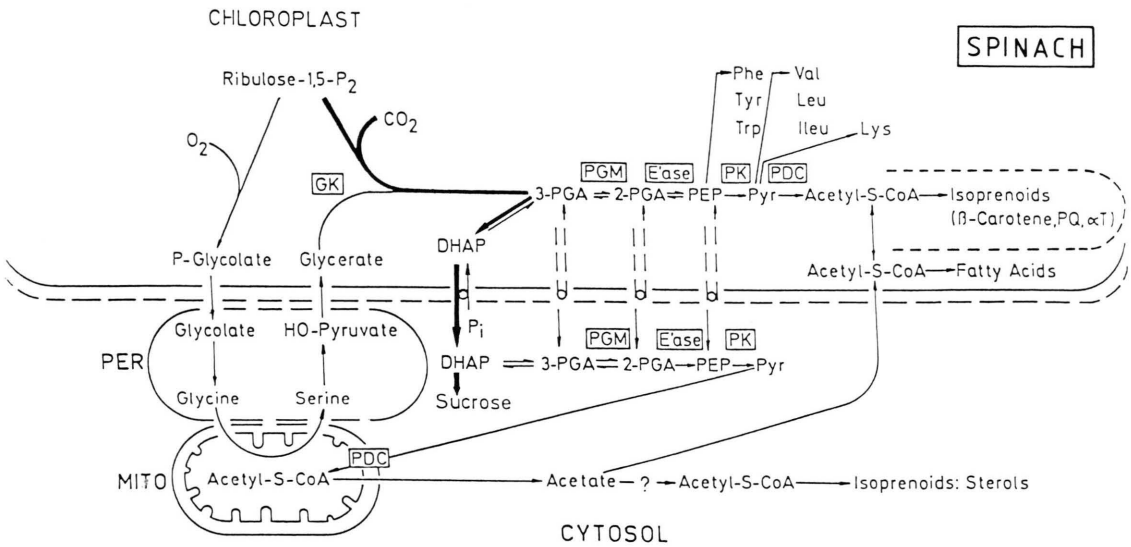

Fig. 5. Proposed scheme of carbon flow from primary photosynthetic products to isoprenoid compounds, fatty acids and other compounds (amino acids, etc.) in spinach chloroplasts. Based on results of this paper and in context with earlier studies [23], the scheme should focus on the following points: (i) 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 artifical ones, the plastidic IPP synthesizing system [64] can also be supported from extraplastidic acetate. The flow diagram in Fig. 5 illustrates the situation in chloroplasts as based on the results for spinach.

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