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Pyruvate-Derived Amino Acids in Spinach Chloroplasts[1]

SYNTHESIS AND REGULATION DURING PHOTOSYNTHETIC CARBON METABOLISM

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ABSTRACT

A probable carbon flow from the Calvin cycle to branched chain amino acids and lipids via phosphoenolpyruvate (PEP) and pyruvate was examined in spinach (*Spinacia oleracea*) chloroplasts. The interpendence of metabolic pathways in and outside chloroplasts as well as product and feedback inhibition were studied. It was shown that alanine, aromatic, and small amounts of branched chain amino acids were formed from bicarbonate in purified intact chloroplasts. Addition of PEP only favored formation of aromatic amino acids. Mechanisms of regulation remained unclear. Concentrations of PEP and pyruvate within the chloroplast impermeable space during photosynthetic carbon fixation were 15 times higher than in the reaction medium. A direct carbon flow to pyruvate was identified (0.1 micromoles per milligram chlorophyll per hour). Pyruvate was taken up by intact chloroplasts slowly, leading to the formation of lysine, alanine, valine, and leucine plus isoleucine (approximate ratios, 100–500:60–100:40–100:2–10). The K_m for the formation of valine and leucine plus isoleucine was estimated to be 0.1 millimolar. Ten micromolar glutamate optimized the transamination reaction regardless of whether bicarbonate or pyruvate was being applied. Alanine and valine formation was enhanced by the addition of acetate to the reaction mixture. The enhancement probably resulted from an inhibition of pyruvate dehydrogenase by acetyl-*S*-coenzyme A formed from acetate, and resulting accumulation of hydroxyethylthiamine diphosphate and pyruvate. High concentrations of valine and isoleucine inhibited their own and each others synthesis and enhanced alanine formation. When pyruvate was applied, only amino acids were formed; when complemented with bicarbonate, fatty acids were formed as well. This is probably the result of a requirement of acetyl-*S*-coenzyme A-carboxylase for bicarbonate.

Not only photosynthesis but also the synthesis of a series of amino acids (19), lipids (10, 31), and isoprenoids (28, 32) has been localized in chloroplasts. Branched chain amino acids derived from pyruvate (valine, leucine, and isoleucine) are of special interest, in protein and to some extent in isoprenoid synthesis (11). The central enzyme in the synthesis of this group of amino acids is the acetolactate synthetase. This enzyme is thought to be exclusively localized in chloroplasts. Miflin (18) demonstrated that acetolactate synthetase cofractionated with marker enzymes of chloroplasts in a sucrose density gradient. The questions addressed in the present study are (a) how far is a plastidic metabolite flow from photosynthetic carbon fixation to pyruvate involved in synthesizing the pyruvate-derived amino acids and other compounds and (b) to what extent is this flow influenced by cytosolic metabolites transported across the envelope membrane.

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MATERIALS AND METHODS

RADIOACTIVE REAGENTS

[^{14}C]Sodium bicarbonate, [1-^{14}C]phosphoenolpyruvate, cyclohexylammonium salt, [1-^{14}C] and [2-^{14}C]pyruvate, sodium salt, [1-^{14}C] and [2-^{14}C] acetate, sodium salt, were purchased from Amersham-Buchler (Braunschweig, West Germany). [1-^{14}C]Palmitic acid, [1-^{14}C]linoleic acid, were generous gifts from Dr. G. Weisser, Institut für Klinische Biochemie, Medizinische Hochschule Hannover, West Germany.

PREPARATION OF CHLOROPLASTS, CHLOROPLAST FRACTIONS, AND OTHER ORGANELLES

(a) Chloroplasts. If not otherwise defined, purified intact chloroplasts were used. For experiments involving labeled bicarbonate and PEP[2] intact chloroplasts were isolated from freshly picked, field grown spinach. For all other experiments, chloroplasts were isolated from garden-grown spinach variety 'Butterfly'. The method for chloroplast isolation in Nakatani and Barber (23) was combined with a purification by Percoll (Pharmacia) gradient centrifugation (12).

Unpurified Chloroplasts. Washed deribbed leaves (0.5–1 kg) were homogenized in isotonic medium pH 6.5 (containing 330 mM sorbitol; 0.2 mM $MgCl_2$; 20 mM Mes-buffer, adjusted with 1 N Tricine to pH 6.5) for 4 × 1 s using a Waring Blendor (all steps at 4°C). The homogenate was filtered through a 20-μm nylon gauze (Vereinigte Seidenwebereien, Krefeld, West Germany) and centrifuged for 1 min at 1,500*g*. The pellet was resuspended in a few ml of isotonic medium pH 7.6 (330 mM sorbitol; 0.4 mM $MgCl_2$; 50 mM Hepes, adjusted with 1 N Tricine to pH 7.6).

Purified Chloroplasts. The chloroplast suspension was placed on a linear 0 to 80% Percoll gradient (prepared from solution (A) dialyzed Percoll, 330 mM sorbitol, 0.4 mM $MgCl_2$, adjusted to pH 7.6 with 1 N HCl and solution (B) isotonic medium pH 7.6). The gradient was run at 3,300*g* for 15 min. The lower band containing the intact chloroplasts was collected at 2,100*g* for 3 min, washed twice in isotonic medium pH 7.6 and resuspended in a small volume of the same medium so that the Chl content was at least 1 mg/ml suspension.

(b) Broken Chloroplasts. Purified chloroplasts were subjected to osmotic shock in a hypotonic buffer solution (containing 10 mM Hepes buffer pH 7.6 and 4 mM $MgCl_2$) for 10 min. Immediately afterwards, substrates were added.

(c) Chloroplast Stroma. The pellet of the purified chloroplasts

[2] Abbreviations: PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; nonreversible GAPDH, nonreversible NADP-D-glyceraldehyde-3-phosphate dehydrogenase; HO-Et-TPP, hydroxyethylthiamine diphosphate; OAA, oxaloacetic acid; PGA, 3-phosphoglycerate; SKA, shikimate.

Table I. *Enzyme Activity of Nonreversible GAPDH (EC.1.2.1.9) and Hydroxypyruvate Reductase (EC.1.1.1.29) In Cell Fractions and Organelles*

	Nonreversible GAPDH	Hydroxypyruvate Reductase
	μmol PGA formed/mg Chl·h	*μmol glycerate formed/mg Chl·h*
Cell homogenate	12.5	292.0
Unpurified chloroplasts	ND[a]	63.0
Purified chloroplasts	ND	7.6

[a] No activity detected.

Table II. *Synthesis of Branched Amino Acids from [2-^{14}C]Pyruvate (0.1 mM) by Chloroplasts, Mitochondria, and Peroxisomes*

Organelle	Amino Acid Formed		
	Ala	Val	Leu + Ile
	nmol/mg protein·h		
Chloroplasts	3.0	0.634	0.390
Mitochondria	74.0[a]	0.030	0.021
Peroxisomes	17.0	0.027	0.040

[a] Including glutamate and aspartate.

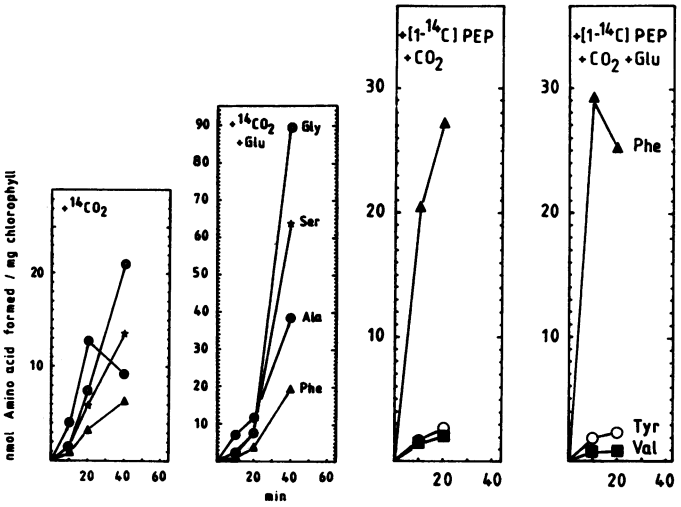

FIG. 1. Time course of amino acid synthesis in chloroplasts (A) from [^{14}C]bicarbonate (10 mM) with and without glutamate (10 μM) (B) [1-^{14}C]PEP, bicarbonate (10 mM) with and without glutamate (10 μM). See "Materials and Methods."

was suspended in a small volume (2.5 mg Chl/ml) of isotonic medium pH 7.6 and was frozen at −20°C for 30 min. It was then slowly thawed at room temperature. The chloroplasts ruptured by freezing were then centrifuged at 175,000*g* for 30 min. The resulting supernatant was used as stroma fraction.

(d) Leaf Homogenate. Washed deribbed leaves (100 g) were homogenized in 20 to 30 ml of isotonic medium pH 7.6 for 1 to 2 min. The filtered suspension was used as leaf homogenate.

(e) Mitochondria and Peroxisomes. Both organelles were isolated from the supernatant of chloroplast preparation centrifuged at 1,500*g*. In order to eliminate membranes of broken chloroplasts, the supernatant was centrifuged at 20,000*g* for 2.5 min. Further fractionation was achieved by centrifugation in a semi-discontinuous sucrose gradient (4, 27). The mitochondrial fraction contained small amounts of Chl similar to Douce *et al.* (7), but no intact chloroplasts. The peroxisomal fraction obtained was free of Chl.

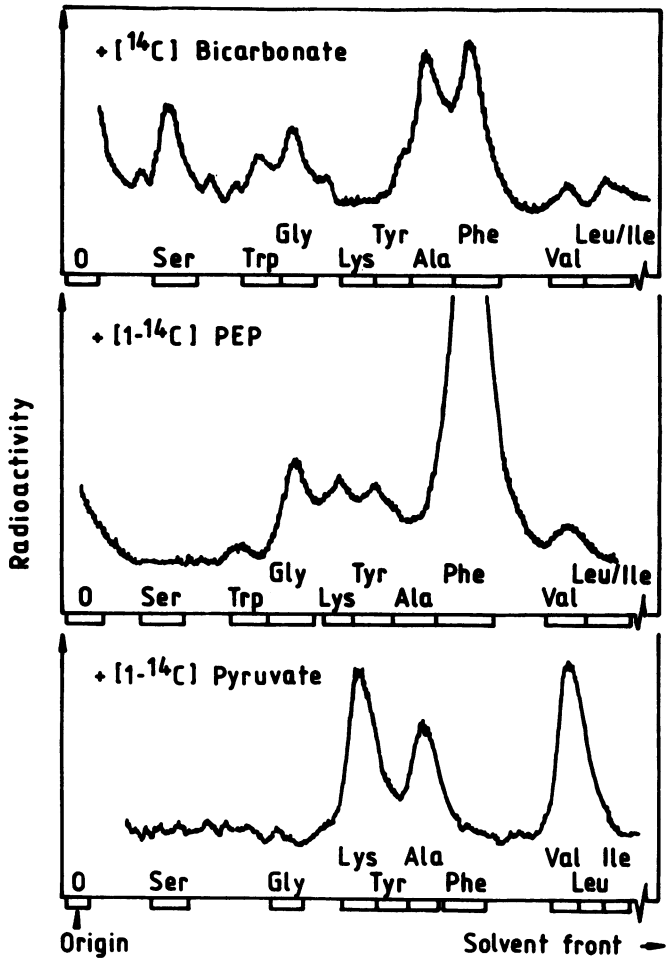

FIG. 2. Radioscan of amino acids formed in chloroplasts after 20 min from [^{14}C]bicarbonate (10 mM); [1-^{14}C]PEP (0.17 mM); and [1-^{14}C] pyruvate (0.01 mM). Amino acids were dansylated and separated by TLC with System I; see "Materials and Methods."

REACTION MIXTURES IN EXPERIMENTS USING CHLOROPLASTS, CHLOROPLAST FRACTIONS, AND OTHER ORGANELLES

If not otherwise specified, the reaction mixture contained either chloroplasts, chloroplast fractions (1 mg Chl/ml), mitochondria, or peroxisomes, suspended in 1 ml of isotonic medium pH 7.6 and substrates. The mixtures were incubated at 20 ± 2°C either in the dark or in the light (0.1 J/cm^2·s; Osram 'Bellaphot'). Aliquots of 0.2 to 0.3 ml were taken at 5, 10, 20, and 40 min after the addition of radioactive substrates. The reactions were terminated as described below.

ASSAY OF AMINO ACIDS

Aliquots (0.2 ml) were transferred into 0.75 ml of a mixture of chloroform:methanol (1:2). Then 0.25 ml chloroform was added and the amino acids were extracted by thoroughly mixing first with 3 ml 0.1 N HCl and second with 0.25 ml methanol plus 3 ml 0.1 N HCl. The aqueous phase was placed on a cation exchange column (Dowex 50 W × 8 in H^+ form, 0.25 × 9 cm) (1). Twenty μg of Ser, Trp, Gly, Tyr, Ala, Phe, Val, Leu, and Ile each were added as carrier substances. After washing with 10 ml water, the amino acids were eluted with 4 ml of 10% NH_4OH. The eluate was evaporated under reduced pressure. The residue was dansylated in 1 ml 0.1 M $NaHCO_3$ plus 1 ml 30% methanol plus 1 ml dansyl-Cl (1 mg/ml acetone) at 40°C for 30 min. Separation of dansyl amino acids was achieved by repeated TLC on silicagel with benzene:pyridine:acetic acid (40:10:1) (system

Table III. *Concentration of PEP and Pyruvate in and outside the Chloroplast*

The reaction was started with 10 mM $NaHCO_3$ in the light.

Time	PEP			Pyruvate		
	Reaction Medium	Chloroplast impermeable space[a]	Chloroplast / Medium	Reaction medium	Chloroplast impermeable space[a]	Chloroplast / Medium
min		*μM*	*ratio*		*μM*	*ratio*
0		45			114	
5	2.4	42	17.5	4.6	105	22.8
10	2.1	43	20.4	7.3	83	11.4
20	3.7	41	11.1	10.3	101	9.8

[a] The Chl contents were 1.90 mg/ml chloroplast suspension; the volume of chloroplast pellet (after centrifugation at 1,500*g*) was 152 μl/ml suspension; the chloroplast impermeable space (calculated according to Heber 1981 [13]) was 72 μl/ml suspension. The external space in the chloroplast pellet (= intramembranous space of envelope plus interorganelle space) was calculated to be 152 − 72 = 80 μl/ml suspension. The calculation of concentrations in the chloroplast impermeable space was based on the assumption that an equal distribution of PEP and pyruvate between impermeable and external space exists.

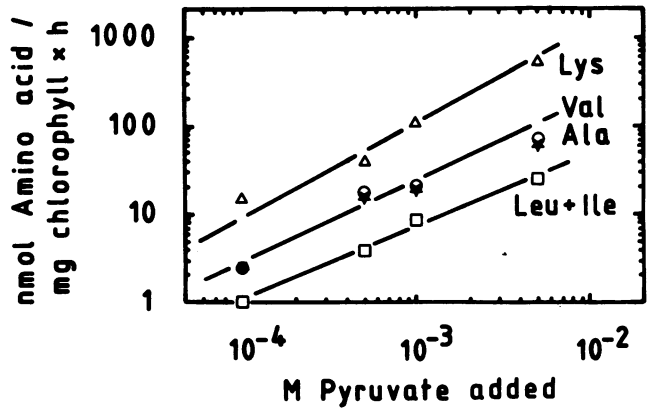

FIG. 3. Amino acid formation by chloroplasts in dependence on concentration of added [2-^{14}C]pyruvate. See "Materials and Methods."

Table IV. *Dependence of Amino Acid Synthesis from [2-^{14}C]Pyruvate (0.1 mM) on Glutamate and/or Acetate in Purified Choroplasts*

	Ala	Val	Leu/Ile
	nmol/mg Chl·h		
Without addition	9.9	6.0	1.63
+ 10 μM Glutamate	15.9	8.3	1.60
+ 0.1 mM Glutamate	11.0	10.6	1.73
+ 10 μM Glutamate + 0.1 mM acetate	17.2	9.7	1.63

I). Dansyl zones were scraped out and the label was counted in 1 ml methanol and 4 ml Hydroluma (Baker) in a liquid scintillation counter (Packard Tricarb 3255). The recovery in the assay was 15%.

Since the recovery of dansylated amino acids was low and the separations of Lys, Ala, Val, and Leu/Ile but not of aromatic amino acids was needed, amino acids were assayed in nonderivatized forms in later studies on pyruvate incorporation. The above residue of NH_4OH-eluate, dissolved in 50% acetone, was subjected to TLC on cellulose with propan-2-ol:water:HCOOH (20:5:1) (system II). Alanine was separated from valine but not leucine from isoleucine. To separate valine from phenylalanine and alanine from tryptophan and tyrosine, the zones from system II were eluted with 2 ml 50% methanol, pH 2, and subjected to rechromatography on silicagel with butanol:water:acetic acid (3:1:1) (system III). Scintillation counting was done as described above. The recovery of amino acids after TLC on system II was 80 to 90%, that in system II plus III was 45 to 55%.

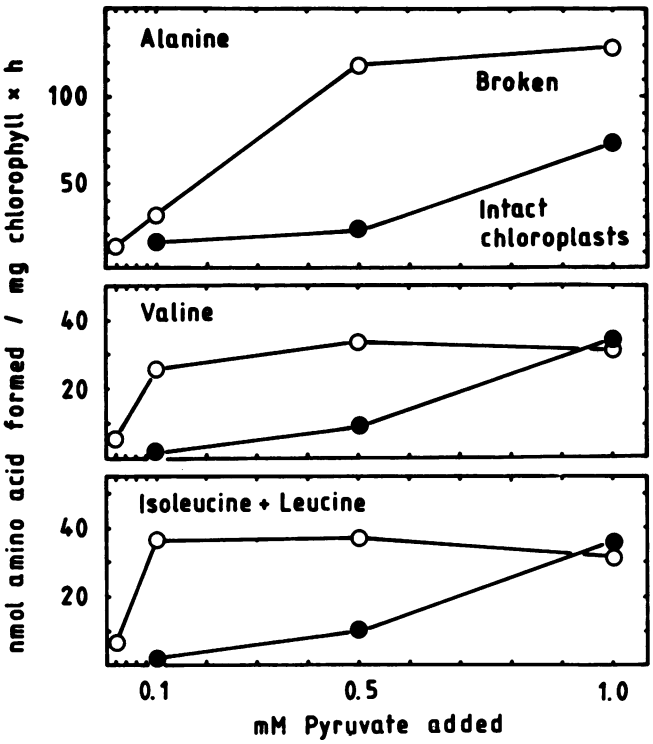

FIG. 4. Amino acid formation from [2-^{14}C]pyruvate in broken and intact chloroplasts. See "Materials and Methods."

To separate leucine from isoleucine, repeated horizontal TLC on silicagel with butanone-2:pyridine:water:acetic acid (70:15:15:2) (system IV) was applied. On such a gel system, isoleucine moves more slowly than leucine. To obtain an optimal fractionation of lysine, TLC on silicagel with ethanol:NH_4OH (34%; 7:3) (system V) was applied.

ASSAY OF FATTY ACIDS FROM ACYLLIPIDS

The chloroplast reaction was terminated by the addition of 2 ml methanol and 2 ml 1 N KOH to 0.2-ml aliquots. Fatty acid dissolved in methanol (120 μg of each) was used as carrier. After refluxing at 80°C for 15 min, the suspension was thoroughly mixed with 2 ml 4 N H_2SO_4 and 1 ml chloroform. The chloroform phase including the washing solution of protein coagulate (0.5 ml chloroform) was evaporated under reduced pressure. Methylesters of free fatty acids and acyl residues of lipids were

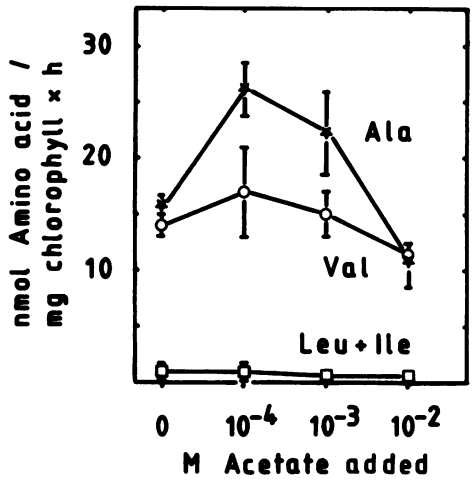

FIG. 5. Effect of acetate on amino acid formation in chloroplast from [2-^{14}C]pyruvate (0.1 mM). See "Materials and Methods."

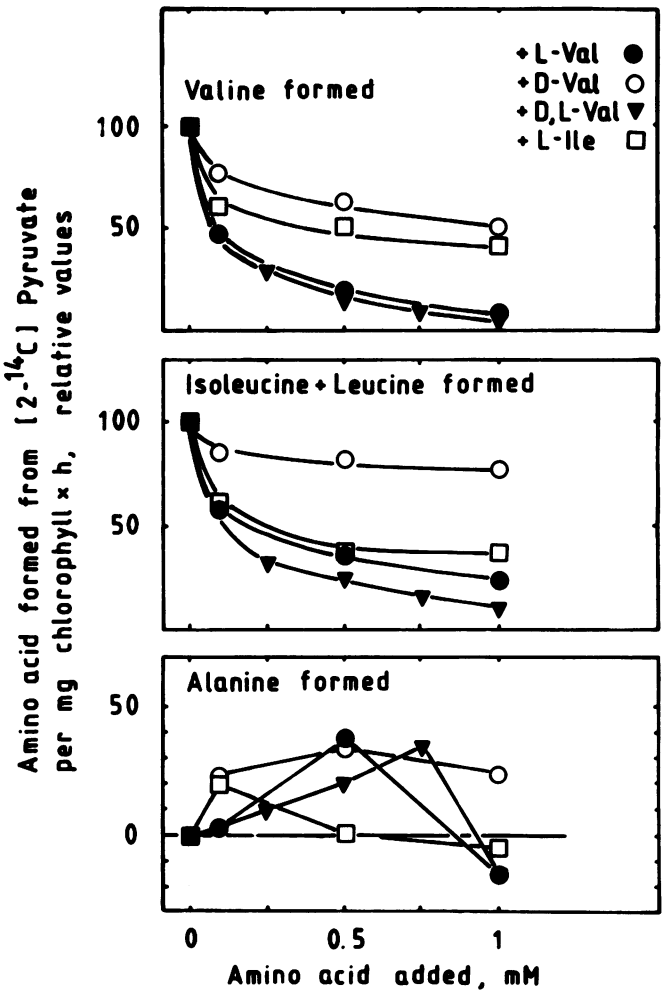

FIG. 6. Influence of added L-, D-, and D,L-valine, or isoleucine, on the formation of valine, isoleucine plus leucine, and alanine from [2-^{14}C] pyruvate (0.1 mM) in chloroplasts. See "Materials and Methods."

formed by heating in sealed ampules in 2 ml methanol:benzene:H_2SO_4 (100:5:5) at 80°C for 60 min (25). After addition of 2 ml water, the esters were extracted twice with 2 ml petroleum ether (b.p. 40–60°C):diethylether (1:1). TLC was performed on silicagel (impregnated with 5% $AgNO_3$ in acetonitrile) with hexane:diethylether (85:15) (system VI). The esters were visualized by spraying with 2,7-dichlorfluorescein.

Table V. *Formation of [^{14}C]Alanine, Valine, Leucine + Isoleucine from [2-^{14}C]Pyruvate (0.1 mM), Acetate (0.1 mM), and Glutamate (10 μM) in Different Cell- and Chloroplast Fractions*

	Ala	Val	Leu + Ile	Total ^{14}C Incorporation of Added [2-^{14}C] Pyruvate after 20 min Incubation
	nmol/mg Chl·h			%
Leaf homogenate	176.7	0.39	0.25	40
Unpurified chloroplasts	139.8	0.39	0.68	34
Purified chloroplasts	42.0	1.32	0.74	23
Chloroplast stroma (175,000*g*)[a]	29.4	0.39	0.21	13
Chloroplast thylakoids	17.9	0.15	0.07	5
Chloroplast stroma plus thylakoids	21.5	0.34	0.20	9

[a] Calculated from the Chl contents of the corresponding chloroplast suspensions.

Table VI. *Effects of Adding of Bicarbonate (5 mM) on the Incorporation of [2-^{14}C]Pyruvate (0.5 mM) into Fatty Acids Compared to Branched Chain Amino Acids in Purified Chloroplasts*

	Pyruvate	Pyruvate + Bicarbonate	Acetate	Acetate + Bicarbonate
		nmol/mg Chl·h		
Ala + Val + Leu + Ile	73.3	65.3	ND[a]	ND
Palmitic and stearic acids[b]	ND	20.0	ND	55.6
Oleic acid[b]	ND	7.2	ND	32.0

[a] Concentration too low for measurement.
[b] Recovery (25%) was determined by radioactive references.

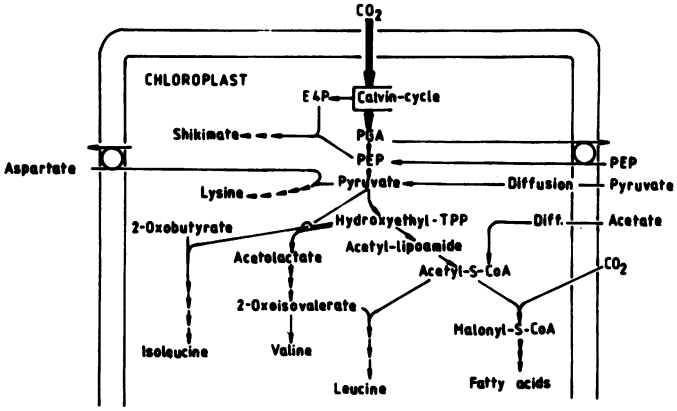

FIG. 7. Proposed scheme of interactions in metabolic flow during pyruvate and acetate conversion by chloroplasts.

Other Methods. CO_2 fixation rate, Chl as well as protein contents were determined as described earlier (4). Enzyme activities of hydroxypyruvate reductase, nonreversible GAPDH, and isocitrate dehydrogenase were assayed according to the methods in Tolbert *et al.* (33), Kelly and Gibbs (16), and Cox and Davies (6), respectively. PEP and pyruvate were determined by standard enzymological methods (2). To separate the reaction medium from intact chloroplasts, the suspension was centrifuged in Eppendorf tubes at 1,500*g* for 1 min; then the protein from the medium and from the chloroplasts was precipitated with TCA.

RESULTS

Criteria of Purification. In order to measure the contamination of the isolated chloroplast preparations by cytosolic and other enzymes, the isolated organelle fractions were tested for the presence of nonreversible GAPDH as a marker enzyme of the cytosol (16), and hydroxypyruvate dehydrogenase as a marker of peroxisomes (33). Suspensions of isolated chloroplasts were essentially free of cytosol and were only slightly contaminated with peroxisomes (about 2–3% of the amount per mg Chl found in the leaf homogenate) (Table I).

Compartmentation of Branched Amino Acid Synthesis. When rates of branched amino acid synthesis in chloroplasts were compared to those in mitochondria and peroxisomes, the high specific activity of chloroplast proteins indicates that no other organelle is involved in this synthesis (Table II). In accordance with Miflin (18), we concluded that chloroplasts are the main site of branched amino acid synthesis. Synthesis of alanine as well as that of glutamate and aspartate is predominately localized in mitochondria. Alanine is also synthesized in peroxisomes at high rates.

Amino Acid Synthesis in Purified, Intact Chloroplasts during Photosynthetic Carbon Fixation. A prerequisite for the synthesis of amino acids in chloroplasts from CO_2 are high photosynthetic CO_2 fixation rates (here 121 ± 19 μmol CO_2/mg Chl·h) and the maintenance of high levels of triosephosphates within the isolated chloroplasts. Thus, the loss in plastidic DHAP caused by the phosphate translocator transport (14, 34) had to be minimized. To lower the DHAP gradient between chloroplasts and suspension medium, chloroplast suspensions were used which contained more than 1 mg Chl/ml. This procedure is suitable for reactions with only a moderate light requirement. An additional improvement for chloroplast syntheses was achieved by minimizing the concentration of Pi in suspension medium to decrease the rate of counter transport by the phosphate translocator (34).

The incorporation of bicarbonate into aromatic amino acids was studied earlier by Bickel *et al.* (1). Alanine and, from the branched chain amino acids, valine were also formed (Figs. 1 and 2). Of the aromatic amino acids, only phenylalanine was quantified. The synthesis of glycine and serine may be attributed to contamination by adhering peroxisomes. Figure 1 also shows that under the present condition the pool of amino acids which donate amino groups in transaminase reactions (20) was sufficient for the formation of only about 100 nmol amino acids/mg Chl·h. When 10 μM glutamic acid was added, the synthesis of amino acids in the chloroplast could be increased to about 300 nmol/mg Chl·h.

Table III leads to the conclusion that low levels of PEP and pyruvate for amino acid formation were synthesized in chloroplasts from PGA of the Calvin cycle (for enzymes involved, see "Discussion"). The concentrations of PEP and pyruvate were lower outside than inside the chloroplast. Multi organelle vesicles did not participate significantly in PEP synthesis because they were present only at low levels in chloroplast suspensions (3–5% [17]).

Preferred Synthesis of Aromatic Amino Acids from Exogenous PEP. When PEP was added to illuminated chloroplasts, aromatic amino acids were formed preferentially (Figs. 1 and 2). Neither alanine nor branched chain amino acids derived from pyruvate were detected in considerable amounts; the reason is unclear. Addition of 10 μM glutamate to the chloroplast preparation increased the rate of phenylalanine synthesis from 80 to at least 115 nmol/mg Chl·h. Tyrosine and tryptophan were synthesized at lower rates (data not shown). The rates of synthesis were largest when 0.1 to 1 mM PEP was added (28).

Synthesis of Branched Chain Amino Acids from Exogenous Pyruvate. When pyruvate was added to chloroplasts, it was transported into chloroplasts at low rates to form alanine, lysine, and the branched chain amino acids valine, leucine, and isoleucine (for fatty acid synthesis, compare Ref. 24).

Branched Chain Amino Acids. Rates of amino acid synthesis continuously increased with the concentration of added pyruvate indicating a reaction controlled by diffusion (Fig. 3). A saturation with pyruvate was not reached in the physiological range. Rates of alanine synthesis comparable to that of the bicarbonate experiments were only obtained by using more than 1 mM exogenously added pyruvate. This may explain the low rates in the case of 0.1 mM pyruvate (Table IV). The rate-limiting role of the pyruvate transfer across the chloroplast envelope membrane was convincingly demonstrated by using chloroplasts which were osmotically shocked in a small volume of hypotonic medium in comparison to the studied intact chloroplasts. As plotted in Figure 4, the K_m of the valine synthesis applying pyruvate was in the 10 μM range, that of the alanine synthesis in the 0.1 mM range. It should be mentioned that shocked chloroplasts were active in the valine synthesis for only 5 to 10 min indicating a loss of cofactor(s) into the suspension medium, etc. Consequently, shocked chloroplasts were only used for this topic. All rates were increased by adding 10 μM glutamic acid (Table IV).

To reduce the conversion of imported pyruvate to acetyl-*S*-CoA by chloroplast pyruvate dehydrogenase (8, 35), acetate was added and found to be optimal in concentrations of 0.1 mM in alanine and valine synthesis (Fig. 5; Table IV).

L-Valine and L-isoleucine in millimolar exogenous concentrations inhibited their own synthesis as well as each others almost completely (D-valine inhibited at a lesser degree) (Fig. 6). This may be caused by feedback regulation of the acetolactate synthetase, a common enzyme of both amino acid syntheses (18). The inhibition of the synthesis by valine had the effect of preserving the added pyruvate. Alanine synthesis was optimally increased by addition of 0.5 mM valine or 0.1 mM isoleucine (Fig. 6).

Further, the effect of purification of chloroplasts on valine synthesis was studied. Highest rates were yielded by using purified chloroplasts (Table V). Unpurified chloroplasts and especially the leaf homogenate based on Chl contents were less active because further pyruvate-consuming reactions are probably competing for the substrate.

The stroma and the thylakoid fraction were less active than intact purified chloroplasts (Table V). The demand for cofactors in the formation of valine from pyruvate will be studied.

The synthesis of valine and leucine plus isoleucine from exogenous pyruvate (0.1 mM) was largely light dependent. The rates in the dark were low (light and dark rates, nmol/mg Chl·h: valine, 2.84 and 0.48; leucine + isoleucine, 0.51 and 0.13). One or few enzymes of their synthesis are probably activated by the light or the rate of reducto isomerase (3) is limited by the availability of NADPH from photosynthesis.

Lysine. Further features of lysine synthesis (already described [21]) became evident in spinach, especially with the variety 'Butterfly'. Rates of lysine formation were strongly enhanced by increasing the exogenous pyruvate concentration. Rates 8 times that of valine were reached by adding 5 mM pyruvate. The low lysine formation under normal conditions may be caused by unfavorable kinetic properties of the enzyme system (Fig. 3). Addition of 1 mM lysine exerts a complete feedback regulation (data not shown) probably by regulation of the aspartate kinase (3). The possibility for the synthesis of lysine via aminoadipinic acid pathway was found false: only [1-^{14}C]- or [2-^{14}C]pyruvate and in no case [5-^{14}C]2-oxoglutarate or [2-^{14}C]acetate were incorporated into lysine (data not shown).

According to Mills and Joy (20), dicarboxylates imported by the dicarboxylate translocator (14) are present in relative high amounts even after chloroplast isolation. As expected, aspartate in chloroplasts was sufficient to achieve a constant rate of synthesis via the diaminopimelate pathway for a period of 40 min.

Effect of Additional Supply of Bicarbonate. If pyruvate was added to illuminated chloroplast suspensions, no or only low amounts of fatty acids could be detected. A decisive hint on the possible reason was given in Sauer and Heise (25) and personal communication from Dr. K. P. Heise that the acetyl-*S*-CoA carboxylase is rate limiting in the fatty acid synthesis and strongly dependent on supply of CO_2. The acetyl-*S*-CoA carboxylase and the ribulose-1,5-bisphosphate carboxylase of the Calvin cycle compete for CO_2 from bicarbonate in illuminated chloroplasts. As shown in Table VI, a considerable fatty acid synthesis occurred only after addition of 1 to 5 mM bicarbonate.

DISCUSSION

Synthesis of Amino Acids during Photosynthesis of Plant Cells Compared to Isolated Chloroplasts. It is well known that about one-third of the photosynthetically fixed carbon is found in amino acids of unicellular algae like *Chlorella* (5) and *Scenedesmus* (29) as well as in protoplasts and multiorganelle vesicles (17) after (about 1 h) exposure to $^{14}CO_2$. Generally, the incorporation occurs into (a) glycine and serine of photorespiration, (b) alanine arising from pyruvate, and (c) aspartate, asparagine, and glutamate, glutamine originating from OAA and 2-oxoglutarate of the tricarboxylate cycle. The last mentioned amino acids are predominately involved in the cell metabolism like transamination reaction (Asp, Glu, Ala) ammonia assimilation (Glu, Gln), nucleic acids synthesis (Glu, Asp), etc. They originate from triosephosphates, predominately DHAP, after transfer across the chloroplast envelope membrane by the phosphate translocator shuttle system (14) and subsequent conversion by the cytosolic glycolysis and mitochondrial tricarboxylate cycle. These amino acids are formed only, if at all, in low amounts in purified chloroplasts during photosynthetic CO_2 fixation due to elimination of corresponding enzymes of the cytosol, mitochondria, and peroxisomes leaving triosephosphate nonmetabolized in the medium (for comparison of purified chloroplasts and multiorganelle vesicles, see Ref. 17).

Branched chain amino acids of the aspartate and pyruvate family, furthermore methionine, lysine, and the amino acids of the shikimate pathway were barely detected by applying classic techniques of CO_2 fixation products because the rates of their synthesis were between 0.01 and 0.1 μmol/mg Chl·h. Some of these amino acids were unambiguously detected by applying the sensitive dansylation technique described in "Materials and Methods." A further proof of the synthesis of amino acids in chloroplasts was obtained by isotope experiments using aspartate for the amino acids of the aspartate family (21) as well as PEP and shikimate for that of the shikimate pathway (1, 28). The isolation of enzymes in chloroplasts is a further proof of the localization of the pathway. Shikimate oxidoreductase/dehydroquinate hydrolyase (SOR/DHQ) as one of the important enzymes of the shikimate pathway has been highly purified (1,300-fold) from chloroplast stroma of spinach. The existence of a cytosolic isoenzyme could be excluded by different techniques including protoplast fractionation (9).

Pyruvate and Acetyl-*S*-CoA Formation in Chloroplasts. The incorporation of bicarbonate, PEP, and pyruvate into amino acids in rates up to 0.1 μmol/mg Chl·h and simultaneously into acyllipids (22, 24) leads to the conclusion that a defined, though only low-dimensioned metabolite flow exists coming from PGA of the Calvin cycle to acetyl-*S*-CoA. Further evidence was obtained that the concentrations of PEP and pyruvate in the chloroplast were about 3 times higher than in the surrounding reaction medium. The studies on enzymes in other laboratories allow the same conclusion. Scheibe and Beck (26) detected a phosphoglycerate mutase plus PEP hydratase in *Pisum* chloroplasts which exhibits an activity of 1.5 μmol/mg Chl·h by using the coupled system with pyruvate kinase. Stitt and apRees (30) demonstrated a PEP hydratase and pyruvate kinase activity in the chloroplast fraction using density gradient centrifugation; however, as yet, the existence of a phosphoglycerate mutase was questioned. Herberts *et al.* (15) demonstrated an active pentosephosphate pathway in chloroplasts. The existence of a pyruvate dehydrogenase in *Pisum* chloroplasts was shown by Elias and Givan (8) and by William and Randall (35). On the other hand, it is evident that acetyl-*S*-CoA is formed in rates up to 2 μmol/mg Chl·h by the chloroplast acetokinase from directly imported acetate (24, 31). The chloroplast and the cytoplasmic metabolic flow influence themselves and each other which might be inferred from the transport of triosephosphate and PEP across the chloroplast envelope membrane by the phosphate translocator shuttle (14) and of pyruvate and acetate by diffusion. The question may be raised how far interactions between the flow of both compartments exist under different *in vivo* conditions. Summarizing the known facts on isolated intact chloroplasts, the metabolic pathways derived from pyruvate are depicted in the following scheme (Fig. 7).

It should be emphasized that the fatty acid synthesis from pyruvate or from acetate in chloroplast demands the simultaneous addition of bicarbonate to form malonyl-*S*-CoA at the acetyl-*S*-CoA carboxylase (see "Results") (25). If bicarbonate is lacking, only branched chain amino acids are formed.

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