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Photosynthetic Carbon Metabolism in Photoautotrophic Cell Suspension Cultures Grown at Low and High CO_2[1]

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ABSTRACT

Photosynthetic carbon metabolism was characterized in four photoautotrophic cell suspension cultures. There was no apparent difference between two soybean *(Glycine max)* and one cotton *(Gossypium hirsutum)* cell line which required 5% CO_2 for growth, and a unique cotton cell line that grows at ambient CO_2 (660 microliters per liter). Photosynthetic characteristics in all four lines were more like C_3 mesophyll leaf cells than the cell suspension cultures previously studied. The pattern of ^{14}C-labeling reflected the high ratio of ribulosebisphosphate carboxylase to phosphoenolpyruvate carboxylase activity and showed that CO_2 fixation occurred primarily by the C_3 pathway. Photorespiration occurred at 330 microliters per liter CO_2, 21% O_2 as indicated by the synthesis of high levels of ^{14}C-labeled glycine and serine in a pulse-chase experiment and by oxygen inhibition of CO_2 fixation. Short-term CO_2 fixation in the presence and absence of carbonic anhydrase showed CO_2, not HCO_3^-, to be the main source of inorganic carbon taken up by the low CO_2-requiring cotton cells. The cells did not have a CO_2-concentrating mechanism as indicated by silicone oil centrifugation experiments. Carbonic anhydrase was absent in the low CO_2-requiring cotton cells, present in the high CO_2-requiring soybean cell lines, and absent in other high CO_2 cell lines examined. Thus, the presence of carbonic anhydrase is not an essential requirement for photoautotrophy in cell suspension cultures which grow at either high or low CO_2 concentrations.

Photoautotrophic cell suspension cultures, in which homogeneous cells are grown with CO_2 as the sole carbon source, have the potential of serving as a model for photosynthesis in a leaf. Unlike a leaf, however, the majority of photoautotrophic cells require elevated levels of CO_2 (1–5%) for growth (11, 17), and the successful establishment of a cotton cell line that grows at low CO_2 (660 μL/L) (32) led us to investigate the differences in photosynthetic carbon metabolism between cotton cells grown at the culture room ambient level of 660 μL/L *versus* 5% CO_2. A comparison was also made with two lines of soybean cells that grow at 5% CO_2, which have been reported previously (10, 22, 23). The results presented here show all four cell lines to have very similar carbon metabolism, much like that found in a C_3 leaf.

[1] Supported in part by a McKnight Foundation Award.

MATERIALS AND METHODS

Photoautotrophic Cell Suspension Cultures

Soybean *(Glycine max)* cells and high CO_2-requiring cotton *(Gossypium hirsutum)* cells were grown at 5% CO_2 in a modified MS medium[2] which contained thiamine and hormones as the only organic compounds (22). Cells were subcultured every 2 weeks. Low CO_2-requiring cotton cells were grown at ambient CO_2, which was 660 μL/L, in a modified MS medium (3, 10), and were subcultured every 4 weeks. Cells were used in experiments 1 to 2 weeks after transfer, and were regularly found to be 90 to 95% viable as determined by phenosafranine staining (30). Prior to use, the cells were washed three times with the appropriate buffer (usually 30 mM Mops, pH 7.0). Cells stored for 4 h in buffer at 11 $\mu E/m^2 \cdot s$ in a test tube or Petri dish showed less than a 10% decrease in the rate of $^{14}CO_2$-fixation.

CO_2 Fixation

Cells (10–20 μg Chl) in 1 mL buffer containing at least 200 IU carbonic anhydrase in sealed glass vials were placed on a shaker in a 30°C water bath. They were illuminated from below (300 $\mu E/m^2 \cdot s$) and flushed with the desired CO_2, O_2, N_2 mixtures for 5 min. $^{14}CO_2$ fixation was initiated by the addition of a trace amount of [^{14}C]$NaHCO_3$ (0.5 μCi, 54 Ci/mol). Reactions were quenched with 0.3 to 0.5 mL 3 N formic acid in methanol. Samples were dried at 65°C and resuspended in 0.2 mL HCl. Dpm were measured by liquid scintillation spectrometry. For experiments at saturating CO_2, reactions were initiated with 210 mM [^{14}C]$NaHCO_3$ (0.6 Ci/mol), bringing the final concentration to 10 mM $NaHCO_3$. Reaction rates were linear with time for at least 10 min and proportional to Chl up to at least 30 μg/mL. In the pulse-chase experiment the vials were flushed with 335 μL/L CO_2, 21% O_2, balance N_2 at 2 L/min after 2 min of ^{14}C-fixation. Control vials without cells showed that 90% of the $^{14}CO_2$ was removed in 3 min. Vials were placed in a clear waterbath and illuminated from the side with a slide projector. CO_2 concentrations were calculated based on a pK_a of 6.3 for HCO_3^-/CO_2.

[2] Abbreviations: MS medium, Murashige-Skoog medium; Caps, 3-[clohexylamino]-1-propanesulfonic acid; PEP, phospho*enol*pyruvate; RuBP, ribulose-1,5-bisphosphate.

^{14}C-Label Distribution

Dried samples were extracted twice with 500 μL H_2O. The filtered solution was applied to 0.5 × 3.5 cm columns of Dowex 50W-X8 (100–200 mesh, H^+ form) and eluted with 2 mL H_2O, then 5 mL 2 N NH_4OH. [^{14}C]-Gly, -Gln, and -Glu eluted in the NH_4OH (B) fraction. The neutral fraction from Dowex 50 was applied to the same size Dowex 1-X8 column (100–200 mesh, formate form) and eluted with 3 mL H_2O, 10 mL 4 N formic acid, 10 mL 10 N formic acid, and 10 mL 4 N HCl. These fractions were designated N, A1, A2, and A3, respectively. [^{14}C]Sorbitol eluted in the N fraction, malate and glucose-1-P in A1, 3P-glycerate in A2, and fructose-1,6-P_2 in A3. Recovery was at least 90%. No additional label eluted in 12 M HCl. Carboxylic acids in the A1 fraction, where indicated, were separated on an HPLC column (Bio-Rad HPX-87H)[3] at room temperature with 4 mM H_2SO_4. Malate was identified by coelution with an authentic sample and by conversion to pyruvate with malic enzyme in 20 mM Hepes (pH 7.2), 1 mM NADP, 0.5 mM $MnCl_2$. Fraction B was further purified in some cases by two-dimensional TLC on Analtech MN300 Cellulose plates (20 × 20 cm) without fluorescent indicator, and autoradiographed (7). Samples were scraped, extracted, and counted (1) except that no Protosol was added. Recovery of ^{14}C applied to the TLC plates was 95 to 100%. Amino acids were identified by comparison with ninhydrin-developed standards. Incubation of a sample with alanine dehydrogenase in 50 mM Caps (pH 10), 1 mM NAD, and 0.1 mM alanine resulted in the complete loss of [^{14}C]alanine. Insoluble material was digested with H_2O_2 and $HClO_4$ (14) or with α-amylase in 0.1 M KOAc (pH 4.8), 55°C, 20 h.

CO_2 Response Curve

Reactions were conducted in specially crafted glass vials in order to ensure rapid equilibration of CO_2 between the aqueous and gaseous phases. The vials were approximately 1.8 cm wide and 0.8 cm high with an 0.8 cm tapered neck to fit septa. Vials containing cells (10 μg Chl) in 1 mL 30 mM Mops (pH 7 0) and 200 IU carbonic anhydrase were illuminated and flushed with 21% O_2 for 10 min. Reactions were initiated by the addition of [^{14}C]$NaHCO_3$ and were quenched after 1.0 min. Solutions were transferred to scintillation vials, dried, and counted as above.

Dark Respiration

Dark respiration was measured with an IR gas analyzer in a temperature-controlled, humidified system (26). Cells (50–300 μg Chl) were suspended in 30 mM Mops (pH 7.0), and at least 200 IU/mL carbonic anhydrase in a small open Petri dish to ensure rapid exchange of CO_2 between the gaseous and aqueous phases. Dark respiration rate was proportional to Chl concentration.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

RuBP Carboxylase Assays

Cells were illuminated at 350 μE/m^2·s unless otherwise indicated for 5 min in a Petri dish containing 30 mM Mops (pH 7.0), and at least 200 IU/mL carbonic anhydrase. The suspensions were swirled to prevent settling and were poured into an illuminated mortar prefrozen with liquid N_2 containing frozen homogenization buffer. The final solution contained approximately 50 mM Bicine (pH 8.0) 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 0.1 mM leupeptin, 0.1 mM antipain, and 1 mM PMSF. Examination under a microscope showed that the cells were completely homogenized. An aliquot was removed for later Chl determination, and the homogenate was centrifuged for 3 s in a microfuge at 4°C. Initial activity was measured immediately by the addition of an aliquot of clarified homogenate to the reaction solution. Total activity was measured after incubating the clarified homogenate for 3 to 5 min at 30°C in a 1:1 dilution with 0.2 mM 6-P-gluconate, 50 mM Bicine (pH 8.0), 40 mM $MgCl_2$, 40 mM $NaHCO_3$. A time course showed that activation was complete. The final reaction solution contained 50 mM Bicine (pH 8.0), 10 mM $MgCl_2$, 10 mM [^{14}C]$NaHCO_3$, and 0.4 mM RuBP in a sealed scintillation vial. Reactions were quenched after 30 s with 0.25 mL 3 N formic acid in methanol. Reactions were linear up to 60 s. Controls with heated cell extract or minus RuBP showed no reaction. Samples were dried and counted as described above.

PEP Carboxylase Assays

Cell homogenate was prepared as described above. Enzyme activity was determined spectrophotometrically at 340 nm in 2.5 mM PEP, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM glucose-6-P, 1 mM DTT, 0.1 mM EDTA, 0.2 mM NADH, and 50 mM Bicine (pH 8.0) and 5 IU/mL malate dehydrogenase. A rapid initial decrease in absorbance was observed upon the addition of extract which was allowed to stabilize before the reaction was initiated with PEP. The rates were calculated after subtraction of the background reaction observed with heated samples. Reactions were conducted in the presence and absence of 16 IU/mL lactate dehydrogenase. Reactions without $NaHCO_3$ were conducted in sealed cuvettes using solutions that had been degassed on ice for 4 h. Addition of PEP and PEP carboxylase showed a small amount of HCO_3 to be present.

Inorganic Carbon

Cellular inorganic carbon concentrations in cells were determined following centrifugation through a 1:2 mixture of SF 96–50 and Versilube F50 (General Electric) silicone oils into 1 M glycine (pH 10) and 0.75% SDS (2). Photosynthesis was measured in 30 mM Mops (pH 7.0) and at least 200 IU/mL carbonic anhydrase. Continuous mixing was required for constant rates of CO_2 fixation. Cell volume was determined using ^3H_2O and [^{14}C]sorbitol (9).

Carbonic Anhydrase

Cells or leaf material were frozen in liquid N_2 and homogenized in 25 mM Barbital (pH 8.3) and 5 mM DTT. Assays

(27) were conducted with several aliquots of extract containing different amounts of Chl because, when greater than 50 μg Chl was present, the calculated activity (IU/mg Chl) decreased.

Immunoblotting

Frozen leaf material and cells were homogenized in 30 mM Tris (pH 9.0), 1 mM PMSF, 14 mM β-mercaptoethanol and were clarified by centrifugation. Prior to homogenization of cotton leaves, 1% SDS was added to prevent protein precipitation. Rabbit polyclonal antibodies to spinach carbonic anhydrase (obtained from C. R. Somerville, Michigan State University) were used to probe nitrocellulose blots of 12.5% SDS-PAGE gels. The spots were developed with goat anti-rabbit IgG conjugated to alkaline phosphatase using indolylphosphate and nitrobluetetrazolium as described in the Protoblot System bulletin (Promega Biotech).

Chl and Protein

Chl was determined after extraction with ethanol (31). Protein concentration was determined with the Bio-Rad protein dye-reagent and crystalline BSA as the standard (4).

RESULTS

Characterization

Cotton cells grown at the culture room ambient CO_2 concentration (660 μL/L)(COT-PA) were physically identical in size and shape to those grown at 5% CO_2 (COT-P), except that the packed cell volume of the COT-PA line was as high as 6 to 8 mL/mg Chl compared to 2 to 3 mL/mg Chl for the COT-P line. Observations with a light microscope indicated that the cells were 35 to 60 μm in diameter and were mostly spherical, with some cells elongated up to 120 μm. Cells were largely vacuolar with chloroplasts studding the periphery. Extracts of both cotton cell lines contained 66 μg Chl/mg protein, compared with 63 and 47 μg Chl/mg protein in the 5% CO_2-requiring soybean cell suspensions SB-P and SB1-P, respectively. The pH of extract from homogenized cells was 5.9 ± 0.2 (n = 4), characteristic of vacuolar pH (15).

Photosynthetic CO_2 fixation at 9 μM CO_2 was maximal from pH 6 to 7.5, with a sharp decrease under more alkaline conditions (data not shown). Gross photosynthesis by COT-PA cells at 10 mM total inorganic carbon saturated at about 300 $\mu E/m^2 \cdot s$, with a maximum photosynthetic rate of 120 μmol CO_2/mg Chl·h (data not shown). CO_2 fixation in the dark was 8% of that in bright light. Based on these results all other experiments were carried out at pH 7.0 and 300 to 350 $\mu E/m^2 \cdot s$ unless otherwise specified.

CO_2 Fixation

Photosynthetic CO_2 fixation rates by healthy cotton cells (COT-PA and COT-P) were 100 to 130 μmol C/mg Chl·h at 10 mM inorganic carbon (pH 7), 30°C. Rates for SB-P soybean cells tended to be lower (60–90 μmol C/mg Chl·h), while rates for SB1-P soybean cells were higher (130–170 μmol C/mg Chl·h). Despite the variations in gross photosynthesis, O_2 inhibition was not statistically different between the four cell lines. The amount of $^{14}CO_2$ fixed at 300 μL/L CO_2 in 50% compared to 2% O_2 was 61% for COT-P cells, $^{14}CO_2$ fixed at 300 μL/L CO_2 in 50% compared to 2% O_2 was 61% for COT-P cells, 62% for COT-PA cells, 71% for SB-P cells, and 69% for SB1-P cells. The CO_2 response of photosynthesis by cotton COT-PA and soybean SB1-P cells at 21% O2 and 30°C showed apparent Michaelis-Menten kinetics, with $K_{1/2}(CO_2)$ values of 37 ± 3 μM and 39 ± 5 μM, and V_{max} values of 115 and 117 μmol C/mg Chl·h, respectively (Fig. 1).

CO_2-Fixing Enzymes

RuBP carboxylase in cotton (COT-PA and COT-P) and the SB1-P soybean cell line was 93 to 94% activated in bright light (Table I) with activation saturating at about 250 $\mu E/m^2 \cdot s$ (Fig. 2). However, in soybean line SB-P the enzyme was only 56% activated at 350 $\mu E/m^2 \cdot s$. The initial activity of RuBP carboxylase in SB1-P cells increased by a factor of 2.5 as the light intensity increased from 10 to 200 $\mu E/m^2 \cdot s$, approaching the total activity. Total activity was constant over the range of light intensities used. In contrast, the initial activity of RuBP carboxylase in the SB-P cells, while increasing with increasing light intensity, remained at only 40% of the total activity even at 1000 $\mu E/m^2 \cdot s$. The total activity was about the same as in SB1-P cells and was also constant in the light. PEP carboxylase activity in the four cell lines ranged from 19 to 22 μmol C/mg Chl·h.

Total RuBP carboxylase activity in dark-adapted SB1-P soybean cells, measured after incubating the extract with CO_2 and Mg^{2+}, decreased 60% after 2 h in the dark (Table II). Initial activity decreased to 40% of the activity in the light

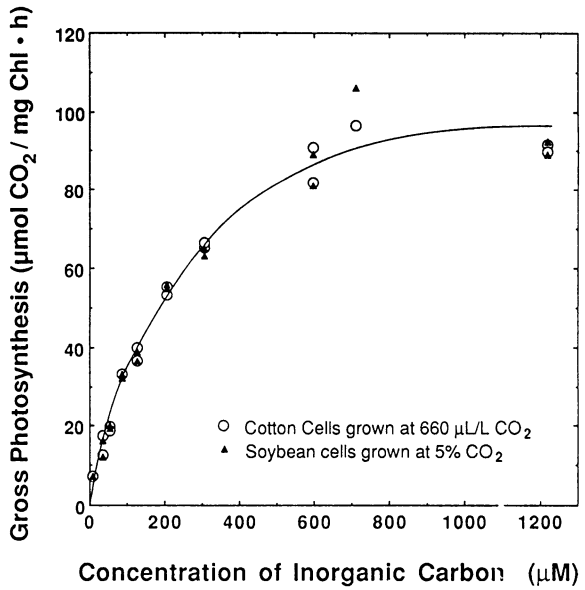

Figure 1. CO_2 response curve of soybean cells grown at 5% CO_2 (SB1-P) and cotton cells grown at 660 μL/L CO_2 (COT-PA). Cells were preilluminated for 10 min in flasks flushed with 21% O_2, balance N_2 in 30 mM Mops (pH 7.0), 30°C in the presence of carbonic anhydrase. Reactions were initiated with $NaHCO_3$.

Table I. *Ribulosebisphosphate and PEP Carboxylase Activities in Cell Suspension Cultures*

Cells were illuminated at 350 $\mu E/m^2 \cdot s$ for 5 min in 30 mM Mops (pH 7.0), equilibrated in air, then frozen in liquid N_2 and homogenized in the presence of protease inhibitors. Initial RuBP carboxylase activity was determined immediately, and total activity after incubation with saturating HCO_3^- and Mg^{2+}.

Cell Line	RuBP Carboxylase		PEP Carboxylase
	Initial activity	Total activity	
	μmol CO_2/mg Chl·h		
Cotton (COT-PA)	151 ± 3[a]	162 ± 2	22 ± 2
Cotton (COT-P)	110 ± 4	117 ± 4	19 ± 2
Soybean (SB-P)	86 ± 3	156 ± 1	19 ± 1
Soybean (SB1-P)	244 ± 3	262 ± 3	19 ± 2

[a] Three samples from the same batch of cells were illuminated in each case.

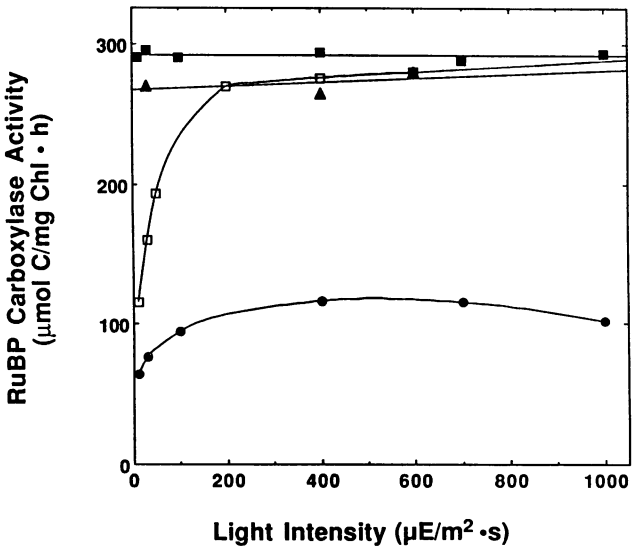

Figure 2. Light activation of RuBP carboxylase in photoautotrophic soybean cells. Cells were illuminated for 5 min at 22 to 24°C in 30 mM Mops (pH 7.0) prior to homogenization in liquid N_2 in 1 mM PMSF, 1 μM leupeptin, and 1 μM antipain. For total activity the extract was incubated in 20 mM $NaHCO_3$, 20 mM $MgCl_2$, and 0.1 mM 6-P-gluconate. (■), SB-P soybean cells, total activity; (▲), SB-1-P soybean cells, total activity; (□), SB1-P soybean cells, initial activity; (●), soybean cells, initial activity.

during the first 6 min of dark incubation, then increased to 60% over several hours. A separate experiment showed that the enzyme was completely reactivated within 10 min in bright light (not shown).

Dark respiration was found to vary in all four cell lines when measured over a 6 month period, but no striking difference was found between cotton cells grown at 660 μL/L CO_2 (COT-PA) compared to those grown at 5% CO_2 (COT-P). Dark respiration rates of COT-PA cells over several months generally ranged from 8 to 13 μmol/mg Chl·h. Typical respiration rates of COT-P cells were 24 μmol/mg Chl·h and for soybean cells SB-P and SB1-P were about 36 and 13 μmol/mg Chl·h, respectively.

Table II. *Dark Inhibition of RuBP Carboxylase in Soybean Cells*

Cells (SB1-P) were incubated in the dark for 2 h in 30 mM Mops (pH 7.0), then frozen, homogenized, and assayed as described in Table I.

Dark Incubation Time	RuBP Carboxylase		Activation (Initial/Total)	Inhibition of Total Activity
	Initial	Total		
min	*μmol C/mg Chl·h*		%	
0	205	235	87	0
6	89	179	50	24
35	103	148	70	37
130	114	133	86	43

Table III. *Distribution of Percent ^{14}C Label in Photosynthetic Products of Photoautotrophic Cell Suspension Cultures*

Cell suspensions in 30 mM Mops (pH 7.0) were illuminated at 30°C and flushed with 330 μL/L CO_2, 21% O_2, balance N_2. Reactions were initiated by the addition of [^{14}C]$NaHCO_3$ and quenched after 2 min. ^{14}C-Labeled compounds were separated by extraction and ion-exchange chromatography into the following fractions: A1, sugar phosphates and organic acids; A2, phosphoorganic acids; A3, sugarbisphosphates; N, neutral compounds; B, amino acids; and I, insoluble compounds.

Cell Line	Growth Conditions	^{14}C in Fractions					
		A1	A2	A3	N	B	I
		%					
COT-PA	Low CO_2	32	6	10	10	34	8
COT-P	High CO_2	32	7	11	10	32	8
SB-P	High CO_2	37	7	8	7	34	7
SB-P	High CO_2	32	7	10	9	32	8

The cotton cell lines (COT-PA and COT-P) and soybean cell lines (SB-P and SB1-P) showed a similar distribution of ^{14}C label among the different fractions separated by ion exchange chromatography, with about one-third of the label in organic acids and sugar monophosphates, one-third in amino acids, and the remaining label nearly equally divided among the PGA, sugar bisphosphate, neutral, and insoluble fractions (Table III). In a separate experiment, the ^{14}C-labeled insoluble material in COT-PA was shown to be at least 95% starch by digestion with α-amylase. In a $^{14}CO_2$ pulse-chase experiment with COT-PA cells at 335 μL/L CO_2, 21% O_2, balance N_2, about 35% of the ^{14}C label was initially in the amino acid fraction. Labeled amino acids increased over 2 min of the chase and then remained relatively constant (Fig. 3). Thin-layer chromatographic analysis of this fraction from a separate experiment showed the initial distribution of ^{14}C to be 69% Gly, 12% Ser, 11% Ala, 7% Asp, and 0.4% Glu. After a 15 min chase the ^{14}C-label in Ser, Ala, and Glu had increased to 30, 20, and 7%, respectively, while Gly and Asp decreased to 38 and 6%, respectively. No other labeled amino acid was detectable in either case.

Prior to the chase, about 37% of the ^{14}C was in the fraction containing organic acids and sugar phosphates, and the label in this fraction decreased continously over the 15 min chase period (Fig. 3). Purification of this fraction by HPLC showed that the majority of label (85%) eluted in the void volume,

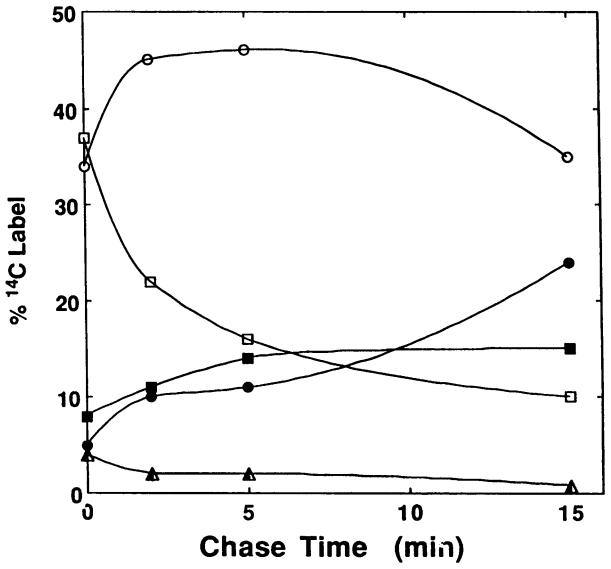

Figure 3. Distribution of ^{14}C in a pulse-chase experiment. Photoautotrophic cotton cells (COT-PA) were grown at 660 μL/L CO_2. Vials containing cells in 30 mM Mops (pH 7.0) 30°C were illuminated and flushed with 335 μL/L CO_2, 21% O_2, balance N_2. Reactions were initiated with [^{14}C]$NaHCO_3$. After 2 min the vials were flushed with 335 μL/L CO_2, 21% O_2, balance N_2. Fractions were separated by ion exchange chromatography. Fractions: (○) B, amino acids; (●) I, insoluble material; (■) N, neutral fraction; (□) A1, sugar phosphates, organic acids; (▲) A2, sugar bisphosphates; (△) A3, phosphoorganic acids.

with 12% of the label coeluting with malate. After a 15 min chase, the percentage label in the void volume had decreased substantially, with the absolute amount in malate increasing slightly (from 6.6–7.9 nCi per 10 μg Chl). The labeled material in the void volume consisted of two compounds that eluted in the region of sugar monophosphates on a Whatman Partisil 10 SAX HPLC column. The labeled compound coeluting with malate was verified as malate by decarboxylation with malic enzyme. Product analysis by HPLC showed no labeled malate remaining; however, 20% of the counts were retained in pyruvate. This indicates that the malate had been multiply labeled, or that some of the label at C_4 of malate had been scrambled with C_1 by fumarase.

Inorganic Carbon

Short-term photosynthesis of COT-PA cells in the presence and absence of external carbonic anhydrase exhibited a lag in the rate of ^{14}C-incorporation for the first 30 s after the addition of [^{14}C]$NaHCO_3$ (Fig. 4). Upon the addition of carbonic anhydrase the lag vanished, implying that CO_2, not HCO_3, is the major form of inorganic carbon that penetrates these cells.

A time course of inorganic and organic carbon accumulation in the COT-PA cell line showed that the organic carbon (acid stable counts) increased linearly while the inorganic carbon (acid labile counts) was essentially constant from 15 to 105 s (Fig. 5). The intracellular concentration of CO_2 was calculated to be about 10 μM in the presence of 9 μM external

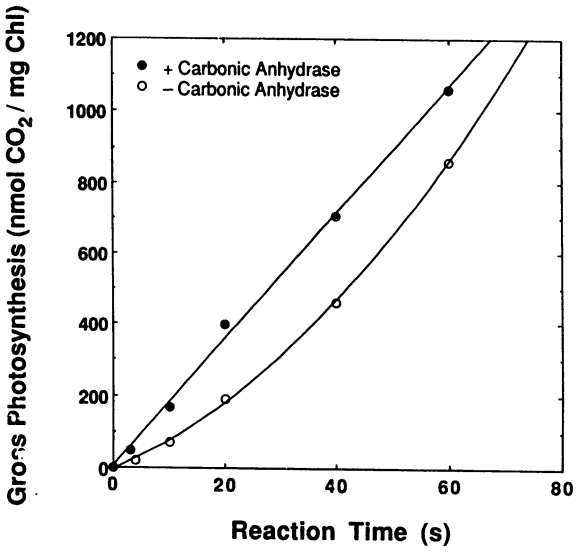

Figure 4. Gross photosynthesis in the presence and absence of external carbonic anhydrase. Photoautotrophic cotton cells grown at 660 μL/L CO_2 (COT-PA) were preilluminated for 5 min in 30 mM Mops (pH 7.0) 30°C with and without 200 IU/mL carbonic anhydrase. Reactions were initiated with $NaHCO_3$ (pH 9.0), to provide a final concentration of 0.2 mM inorganic carbon.

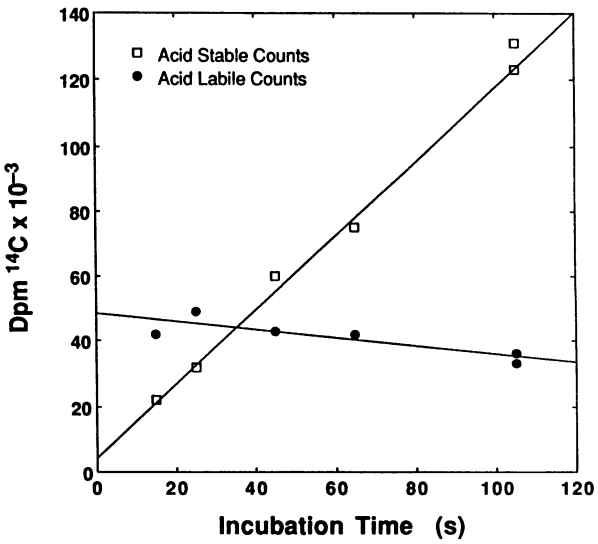

Figure 5. Inorganic carbon in photoautotrophic cotton cells grown at 660 μL/L CO_2 (COT-PA). Preilluminated cells were suspended in 30 mM Mops (pH 7.0), 21°C in the presence of carbonic anhydrase. Reactions were initiated with [^{14}C]$NaHCO_3$ and quenched after a given incubation time by centrifugation through silicone oil into 1 M Gly (pH 10), 0.75% SDS.

CO_2, based on a cellular volume (^3H_2O minus [^{14}C]sorbitol space) of 3.37 mL/mg Chl.

Carbonic Anhydrase

Carbonic anhydrase levels in all four cell lines were examined both by immunoblotting and a standard biochemical

assay in veronal buffer. The antibody for the enzyme from spinach leaf was found to cross-react with proteins in leaf extracts from cotton and soybean. Both soybean cell lines tested positive for carbonic anhydrase, but no carbonic anhydrase was found in cotton cells grown at 5% CO_2 (COT-P) or at ambient CO_2 (COT-PA) (Table IV). We estimated that the cotton cells contained less than 1/200th of the carbonic anhydrase activity present in a spinach leaf. Activity in both spinach leaves and soybean SB1-P cells was in the soluble fraction, and was not associated with the membranes. Activity was completely inhibited by 10 μM ethoxzolamide or by steaming the extract for 3 min.

DISCUSSION

This research was initiated in an attempt to determine any features distinguishing carbon metabolism in the photoautotrophic cells that grow at low CO_2 compared to cells that grow at only high CO_2. The results reported here show that photosynthetic carbon metabolism is identical insofar as it was investigated. Differences in any long-term effects, such as starch degradation or export of organic carbon, were not examined.

Many of the characteristics of the photoautotrophic cell suspension cultures examined here are very similar to those found in C_3 leaves and leaf cells. These cells are therefore potentially a useful model for photosynthesis in leaves. These results contrast with previous reports of carbon metabolism in a number of different photoautotrophic cell cultures, all of which contained much lower levels of RuBP carboxylase, a high fraction of ^{14}C-labeled C_4 products, or both lower RuBP carboxylase activity and more C_4 acids (12, 13, 16, 18).

The CO_2 response of low CO_2-requiring cotton cells and high CO_2-requiring soybean cells demonstrated that most of the CO_2 was fixed by RuBP carboxylase (Fig. 1). The half-saturating level of CO_2, 40 μM at 21% O_2, was similar to that reported for soybean leaf cells (24). The presence of any inorganic carbon concentrating mechanism would have decreased this value, so it is unlikely that these cells concentrate CO_2 internally.

The ratio of RuBP carboxylase to PEP carboxylase (Table I) was similar to that in leaf tissue and much higher than ratios reported for other photoautotrophic cell cultures, where RuBP carboxylase was, at most, twofold higher than PEP carboxylase (8, 20). In the previous studies with photosynthetic cell cultures it was proposed that the low levels of RuBP carboxylase present were analogous to the low levels observed in developing leaves (13, 32). The reason for the difference in RuBP carboxylase levels in the different cell lines is unknown, but it is evident that all four of the cell suspensions studied in detail here are much more like cells in mature leaves.

Table IV. *Carbonic Anhydrase in Photoautotrophic Cell Suspension Cultures*

The presence (+) or absence (−) of the enzyme was determined by both immunoblotting with antibody to spinach leaf enzyme and by the standard biochemical assay.

Sample	Immunoblot	Activity
Spinach leaf	+	3500 IU/mg Chl
Cotton leaf	+	Not determined
Soybean leaf	+	Not determined
Cotton cells (COT-PA)	−	0
Cotton cells (COT-P)	−	0
Soybean cells (SB-P)	+	680 IU/mg Chl
Soybean cells (SB1-P)	+	2670 IU/mg Chl

RuBP carboxylase showed normal light activation at air levels of CO_2 in three of the four cell suspension cultures studied (Table I; Fig. 2). However, in the SB-P soybean cell line, RuBP carboxylase was only approximately half activated at high light. The enzyme itself appeared to be normal in that it could be fully activated with Mg^{2+} and CO_2, and the oxygen inhibition of CO_2 fixation was the same as in the other cell lines. In the dark, total RuBP carboxylase activity decreased in soybean cell line SB-P (Table II), suggesting that these cells synthesize the dark inhibitor, carboxyarabinitol-1-phosphate, as found in soybean leaves (25).

The distribution of ^{14}C-label also supports the major role of RuBP carboxylase in fixing carbon in these cell suspension cultures. As shown in Table III and Figure 3, the vast majority of the label is in products typical of C_3 photosynthesis. A relatively small amount of label was in malate, which turned over very slowly. This is in contrast to the results reported for several other photoautotrophic cell suspensions, all of which had a much higher proportion of the label in C_4 compounds (12, 13, 16, 18).

Photorespiration is a key indicator of C_3 photosynthesis and was found to occur in the suspension cell cultures. Significant levels of ^{14}C-labeled glycine and serine were produced during photosynthetic CO_2 fixation. Also, the magnitude of O_2 inhibition of CO_2 fixation was similar to that seen in leaves of C_3 plants (5). This was not unexpected because the majority of the CO_2 is assimilated by RuBP carboxylase as shown by the distribution of ^{14}C-label. Based on the kinetic properties of RuBP carboxylase in soybean leaf cells (24) it is likely that no photorespiration occurred under the elevated CO_2 conditions used for growth of the high CO_2-requiring cell suspension cultures.

Dark respiration in the cell suspensions was found to be variable. This may reflect, in part, the health of the cells or the stage of the growth cycle (22). Dark respiration rates were generally higher than the 6 to 7 μmol C/mg Chl·h reported for a soybean leaf (19). Similarly, a 7- to 10-fold higher rate of oxygen uptake has been reported for photoautotrophic cells compared to cells isolated from a tobacco leaf (29). This increase in respiration may reflect a more rapid cell division in the cell suspension, as is seen in developing leaves where the values decrease from about 100 μmol C/mg Chl·h to 11 μmol C/mg Chl·h in unfolding to mature leaves (6). However, the cell suspensions were grown under continuous light and the contribution of mitochondrial respiration in the light remains unclear.

Historically, high CO_2 has been necessary to support photoautotrophic cell growth. It was suggested (16) that any cell suspensions capable of growing at low CO_2 must have an inorganic carbon concentrating mechanism similar to that found in algae. As shown here, the cotton cell line which grew at 660 μL/L CO_2 did not possess a CO_2 concentrating mechanism. The lack of an inorganic carbon pump is shown most

directly in Figure 5, where internal inorganic carbon rapidly equilibrated to about 10 μM at an external CO_2 concentration of 9 μM. This observation is inconsistent with the accumulation of higher levels of inorganic carbon found when a similar experiment was conducted with low CO_2-requiring *Chlamydomonas reinhardtii*, which does have a CO_2 concentrating mechanism, and the absence of CO_2 accumulation in a *Chlamydomonas* mutant with a deficiency in the CO_2 concentration mechanism (28). Further, there is not a bicarbonate pump since the cells required CO_2, not HCO_3^- for photosynthesis (Fig. 4). Likewise, the magnitude of the oxygen inhibition of photosynthesis described above would not occur if the internal concentration of CO_2 were elevated.

One point that has been clarified by the results presented here concerns the possible role of carbonic anhydrase in aiding cells to grow at low CO_2. It was reported that photoautotrophic cells grown at 1% CO_2 had less than 10% of the carbonic anhydrase normally present in leaves (29), suggesting that the inability of the cells to grow at low CO_2 was due at least in part to the lack of the enzyme. However, the low CO_2-requiring cotton cells studied here contained no detectable carbonic anhydrase by either the standard biochemical assay or by an immunological probe of soluble cell extracts (Table IV).

The role of carbonic anhydrase in leaves has remained a source of speculation. The most commonly proposed roles are in regulating pH, in supplying CO_2 to RuBP carboxylase, or in aiding diffusion by increasing the CO_2 gradient (21). The enzyme is clearly not required for maximal photosynthesis in the photoautotrophic cotton cells that grow at low CO_2. More significantly, the CO_2 response of the cell lines that did and did not contain the enzyme are identical (Fig. 1). It is therefore unlikely that carbonic anhydrase plays a major part at the chloroplast level in either supplying CO_2 or removing H^+. These results suggest that any requisite role is in dealing with transitory changes not found under the growth conditions of these cells, or that the enzyme is necessary only when the morphology of the leaf makes diffusion more problematic.

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