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Effects of Acetate on Facultative Autotrophy in *Chlamydomonas reinhardtii* Assessed by Photosynthetic Measurements and Stable Isotope Analyses[1]

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The green alga *Chlamydomonas reinhardtii* can grow photoautotrophically utilizing CO_2, heterotrophically utilizing acetate, and mixotrophically utilizing both carbon sources. Growth of cells in increasing concentrations of acetate plus 5% CO_2 in liquid culture progressively reduced photosynthetic CO_2 fixation and net O_2 evolution without effects on respiration, photosystem II efficiency (as measured by chlorophyll fluorescence), or growth. Using the technique of on-line oxygen isotope ratio mass spectrometry, we found that mixotrophic growth in acetate is not associated with activation of the cyanide-insensitive alternative oxidase pathway. The fraction of carbon biomass resulting from photosynthesis, determined by stable carbon isotope ratio mass spectrometry, declined dramatically (about 50%) in cells grown in acetate with saturating light and CO_2. Under these conditions, photosynthetic CO_2 fixation and O_2 evolution were also reduced by about 50%. Some growth conditions (e.g. limiting light, high acetate, solid medium in air) virtually abolished photosynthetic carbon gain. These effects of acetate were exacerbated in mutants with slowed electron transfer through the D1 reaction center protein of photosystem II or impaired chloroplast protein synthesis. Therefore, in mixotrophically grown cells of *C. reinhardtii*, interpretations of the effects of environmental or genetic manipulations of photosynthesis are likely to be confounded by acetate in the medium.

The green alga *Chlamydomonas reinhardtii* is a facultative "acetate flagellate" capable of growing heterotrophically on acetate, but not on Glc or other related carbon sources (Harris, 1989). Many studies of light stress and light regulation of photosynthetic gene expression have been carried out with acetate-grown cells (e.g. Ohad et al., 1990; Danon and Mayfield, 1991; Drapier et al., 1992). Therefore, we were interested in the potential effects acetate may have on photosynthesis and related processes. Acetate is metabolized to triose following ATP-dependent entry into the glyoxylate cycle, whereas inorganic carbon is reduced to triose during photosynthesis. Acetate metabolism may exert opposing influences on utilization of inorganic carbon. Previous studies have reported that acetate transiently inhibits photosynthesis (Endo and Asada, 1996) and stimulates respiration in light-grown cells of *C. reinhardtii* bubbled with air (Fett and Coleman, 1994; Endo and Asada, 1996), possibly via increased alternative oxidase activity (Weger et al., 1990a, 1990b). The ability of acetate to induce isocitrate lyase, the key glyoxylate cycle enzyme necessary for its utilization, is attenuated in the presence of light and inorganic carbon (Martinez-Rívas and Vega, 1993). Conversely, acetate represses expression of nuclear-encoded chloroplast proteins involved in light harvesting and inorganic carbon fixation (Goldschmidt-Clermont, 1986; Kindle, 1987). Thus, mixotrophically grown cells of *C. reinhardtii* may respond differently to light stress than photoautotrophically grown cells, potentially confounding interpretation of responses to genetic and environmental manipulations.

A particularly appropriate tool for studying biomass partitioning is stable isotope ratio mass spectrometry. Estep and Hoering (1980, 1981) determined the fraction of reduced biomass resulting from photosynthesis during mixotrophic growth of *Chlorella sorokiniana* on Glc or acetate medium in the presence of 1% CO_2 using stable hydrogen isotope analysis. Differences in $\delta^{13}C$ have also been used to determine the time of onset of autotrophy in developing seedlings (Deléens et al., 1984; Maillard et al., 1994a, 1994b). Dual isotope methods have been applied to assess carbon and nitrogen allocation during maize stem elongation (Cliquet et al., 1990) and biomass derived from translocated Suc and photosynthesis in the partially photosynthetic hypsophylls (husks) of maize (Yakir et al., 1991) and in vitro-grown potato plantlets (Wolf et al., 1998). Additionally, the effect of acetate on respiratory pathway partitioning can be assessed by on-line analysis of stable $^{18}O_2$ discrimination (Weger et al., 1990a, 1990b; Ribas-Carbo et al., 1995).

The proportions of biomass attributable to photosynthetic CO_2 assimilation and to heterotrophic respiration of a reduced carbon source in mixotrophically cultured algal cells can be estimated from stable isotope determinations using the following equation (modified after Cliquet et al.,

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1990), provided the isotopic signatures of the two sources of carbon are sufficiently different:

Photosynthetic fraction of carbon biomass=

$$(\delta^{13}C_{hetero} - \delta^{13}C_{mixo})/(\delta^{13}C_{hetero} - \delta^{13}C_{auto})$$

This quantitative relationship prevails because: (a) uptake and respiration of reduced carbon substrates result in comparatively little discrimination (about 1‰) relative to the source (DeNiro and Epstein, 1976), and (b) photosynthetic CO_2 fixation is an irreversible process and therefore subsequent biochemical events have only small effects on the $\delta^{13}C$ value (O'Leary, 1988). Results presented in this paper show that the presence of acetate during growth in saturating light and CO_2 inhibits photosynthesis and autotrophic carbon assimilation in wild-type *C. reinhardtii*. This effect was exacerbated in wild-type *C. reinhardtii* grown under low irradiance or in air, and by site-specific chloroplast mutations that predispose *C. reinhardtii* to photoinhibition.

MATERIALS AND METHODS

Strains

Cultures of wild-type (CC-125, 137C *mt*$^+$), a non-photosynthetic *psbA* deletion mutant (CC-744, *ac-u-β mt*$^+$), and a respiration-deficient mutant of *Chlamydomonas reinhardtii* lacking cytochrome oxidase activity (CC-314, *dk-97 mt*$^-$) described by Harris (1989) were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-resistant transformant *dr* (CC-2827) originated from biolistic bombardment of CC-125 with a cloned 10-kb *Bam*HI-*Bgl*II fragment of chloroplast DNA containing the *psbA* gene from the herbicide-resistant *DCMU-4* mutant (Erickson et al., 1984) bearing a Ser-264 to Ala change in the D1 protein of photosystem II (PSII). The *spr/sr* strain (CC-2811) is impaired in chloroplast protein synthesis as a consequence of two single antibiotic-resistance point mutations (A_{474}-> C, A_{1123}-> G) in the chloroplast-encoded 16S rRNA gene (Harris et al., 1989; Heifetz et al., 1997). This strain was obtained by biolistic transformation of CC-125 with a cloned 7.0-kb *Bam*HI chloroplast DNA fragment containing the mutant 16S rRNA gene and most of the 23S rRNA gene proximal to the intron near the 3′ end of this gene.

Growth Conditions

Cells were grown in liquid cultures shaken and bubbled with 5% (v/v) CO_2-enriched air at 25°C under continuous illumination from cool-white fluorescent lamps under low (<25 μmol m^{-2} s^{-1}), moderate (350 μmol m^{-2} s^{-1}), or high (600 μmol m^{-2} s^{-1}) photosynthetically active radiation (400–700 nm). High-salt minimal medium (HS) was used for photoautotrophic experiments, whereas mixotrophic and heterotrophic growth were carried out in either high-salt acetate medium (HSHA) containing 29.4 mM sodium acetate or in Tris-acetate phosphate (TAP) buffer containing 17.5 mM acetate (Harris, 1989). Liquid cultures were maintained in the early- to mid-exponential growth phase by periodic dilution for several days to ensure acclimation to the growth environments. Aliquots of these cultures were used to inoculate 250- to 300-mL liquid cultures into 500-mL baffled shake flasks (Bellco, Vineland, NJ) at 2×10^5 cells mL^{-1}, or were spread onto 1.5% agar plates of the same medium for analysis. The pH of liquid cultures in HS, HSHA, and TAP medium bubbled with 5% CO_2 remained within the range 6.6 to 7.4. Cultures on agar plates supplemented with 5% CO_2 were placed inside a closed plexiglass chamber and supplied with mixed gas at a flow rate of approximately 500 cm^3 min^{-1}, while those at ambient CO_2 levels (in air) were maintained on lighted shelves at 25°C.

Measurement of Photosynthesis, Respiration, and Growth Rates

Cells for photosynthesis measurements were grown under high light and bubbled with 5% CO_2 in cultures of HS supplemented with 0, 3.7. 7.4, 14.7, and 29.4 mM sodium acetate to the early exponential phase (A_{750} = approximately 0.1), gently pelleted, and resuspended (A_{750} = 0.175) in fresh growth medium with 10 mM $NaHCO_3$. Respiration, maximum rate of net photosynthetic O_2 evolution, and chlorophyll fluorescence quenching were measured at growth temperature under 300 and 600 μmol m^{-2} s^{-1} red actinic light, as described by Heifetz et al. (1997).

The incorporation of $^{14}CO_2$ into acid-stable products was measured under high light in 1.5-mL aliquots of cells in 40-mL centrifuge tubes (Corex, Corning, NY) containing 0.5 mL of a bicarbonate reaction mixture (0.2 M Tris, pH 8.0, 40 mM $NaHCO_3$, 4 μCi $NaH^{14}CO_3$ [6.6 Ci/mol, NEN Life Science Products, Boston]) and a 1-cm magnetic stir bar. Cells were stirred continuously, and 0.5-mL aliquots were removed after 6, 12, and 18 min and placed in scintillation vials with 500 μL of 1 N HCl to drive off the unincorporated ^{14}C. Duplicate 100-μL aliquots from each sample were counted in 10 mL of EcoLume (ICN, Costa Mesa, CA) scintillation fluid. Rates of ^{14}C incorporation into acid-stable products were linear for all samples over the 18-min assay period.

Chlorophyll content and exponential growth rates (cell/biomass doubling times) were calculated as described previously (Lers et al., 1992; Förster et al., 1997).

[^{13}C]Acetate Labeling

The $\delta^{13}C$ of acetate samples from eight different suppliers ranged from −44.1 to −19.5‰. Laboratory compressed air ($\delta^{13}C$ approximately −8‰) was mixed with bottled CO_2 from various sources to produce 5% CO_2 in which the $\delta^{13}C$ varied from −44‰ to +4‰ between different experiments. The dynamic range of the isotope discrimination assay for TAP-grown cells was expanded by supplementing the naturally available ^{13}C from acetic acid with 2 mg L^{-1} 1,2 [^{13}C]acetate (Sigma-Aldrich, St. Louis, catalog no. 28,201–4). Thus, the span of $\delta^{13}C$ of wild-type cultured in the dark or CC-744 cultured in dim light on TAP medium ranged from −21‰ (photoautotrophic growth on CO_2) to >110‰ (heterotrophic growth on ^{13}C-TAP), permitting a very ac-

curate estimation of the photosynthetic fraction of carbon assimilated under mixotrophic conditions.

Sample Preparation for Carbon Isotope Mass Spectrometry and $\delta^{13}C$ Determinations

Cells were harvested from liquid cultures in the mid-exponential phase (approximately 3×10^6 cells mL^{-1}) by centrifugation at room temperature, washed three times in double-deionized H_2O, pelleted in 1.5-mL microcentrifuge tubes at 4°C, and stored at −70°C until lyophilization. Cells grown for 5 to 10 d on agar plates were transferred directly to microcentrifuge tubes with sterile inoculating loops, avoiding transfer of agar substrate ($\delta^{13}C$ = −17‰ to −19‰). Lyophilized samples were ground finely, and aliquots (200–2,000 μg) were weighed into tin capsules and combusted in an automated elemental analyzer (NA1500, Carlo Erba, Milan) for determination of $^{13}C/^{12}C$ ratios using a stable isotope ratio mass spectrometer (VG Isogas SIRA II, Middlewich, UK) (Yakir et al., 1991). Values are reported as means ± SE of duplicate or triplicate samples as indicated in the figures and tables.

On-Line Respiratory ^{18}O Fractionation

The on-line sample trapping and preparation system used for liquid-phase $^{18}O_2$ discrimination during respiration was that described by Ribas-Carbo et al. (1995). Aliquots of exponentially growing liquid cultures (0.04 to 0.1 A_{750} = 0.5 to 2×10^6 cells mL^{-1}) were transferred to a 30-mL capacity cylindrical plexiglass chamber connected to a vacuum trapping line and fitted with an O-ring-sealed plunger to facilitate sampling without the introduction of air bubbles. Dissolved gases were sparged from 5-mL aliquots of the cell suspension sampled at 8- to 20-min intervals by bubbling with He, and the O_2 was trapped at 77°K on a molecular sieve after removal of CO_2 and H_2O in a vacuum line. Cells treated with 10 μM DCMU to inhibit photosynthetic O_2 evolution gave the same results as experiments using a darkened chamber (data not shown). Oxygen isotope discrimination was calculated as described in Ribas-Carbo et al. (1995) using an Ar/N_2 ratio of 0.0388 to account for the aqueous diffusivities of these gases. Slope SEs were adjusted for sample size (Weger et al., 1990b) and *F* tests of significance were used for pairwise comparisons of regression slopes.

RESULTS

Effect of Acetate Concentration on Photosynthesis and Growth Rate in Saturating Light and CO_2

Maximum rates of net O_2 evolution and CO_2 incorporation into acid stable products by wild-type cells of *C. reinhardtii* declined with increasing acetate concentration in the mixotrophic growth medium under high (saturating) light and CO_2 conditions (Table I). HSHA, which contains 29.4 mM acetate, effected a 48% reduction in the maximum rate of O_2 evolution and a 56% reduction in CO_2 fixation rate. The lowest acetate concentration tested (3.7 mM) reduced O_2 evolution and CO_2 fixation by 26% and 34%, respectively. In contrast, the growth rate, respiration, PSII efficiency, and chlorophyll content were not affected by acetate concentration.

Evaluation of Isotopic Fractionation during Heterotrophic and Photoautotrophic Growth of *C. reinhardtii*

The assessment of the relative contributions of photosynthetic CO_2 fixation and respiration of acetate to cell metabolism during mixotrophic growth first requires baseline isotopic signatures of cells grown heterotrophically and photoautotrophically. The $\delta^{13}C$ value of heterotrophically grown wild-type cells (data not shown) and cells of a nonphotosynthetic *psbA* deletion mutant (CC-744) grown in dim light on HSHA (Fig. 1A) was strongly correlated with the $\delta^{13}C$ of the acetate present in the growth medium. These results demonstrate that heterotrophic metabolism of acetate by *C. reinhardtii* results in little or no carbon isotope discrimination. The $\delta^{13}C$ values of wild-type *C. reinhardtii* biomass grown photoautotrophically under saturating light and CO_2 remained relatively constant throughout the exponential portion of the growth curve and increased only slightly in the early stationary phase (Fig. 1B). Growth of wild-type *C. reinhardtii* in HS liquid

Table I. *Effect of acetate concentration on growth rate and photosynthesis of wild-type C. reinhardtii in the early log phase when grown and analyzed under saturating light (600 mmol m^{-2} s^{-1}) in the presence of 5% CO_2*

Photosynthesis values are expressed in absolute units and as a percentage of the control HS culture without acetate.

Acetate	Growth Rate[a]	Respiration[b]	Net Photosynthesis[b]		F_v/F_m	Chlorophyll[c]
		O_2 uptake	O_2 evolution	^{14}CO fixation		
mM			μmol mg^{-1} Chl h^{-1}			mg/A_{750}
0	149 ± 0.005	−79	345 (100)	465 (100)	0.71	28 ± 3
3.7	0.162 ± 0.005	−93	256 (74)	308 (66)	0.66	28 ± 3
7.4	0.153 ± 0.005	−94	226 (66)	360 (78)	0.69	28 ± 2
14.7	0.153 ± 0.005	−77	206 (60)	257 (55)	0.70	29 ± 3
29.4	0.148 ± 0.005	−87	180 (52)	207 (44)	0.70	28 ± 2

[a] Mean ± SE from five independent experiments. [b] Data from single representative experiments measuring O_2 consumption, O_2 evolution, or $^{14}CO_2$ fixation. Replicate experiments show the same relative reductions in both parameters with increasing acetate concentration in the growth medium. [c] Mean ± SE from three independent experiments.

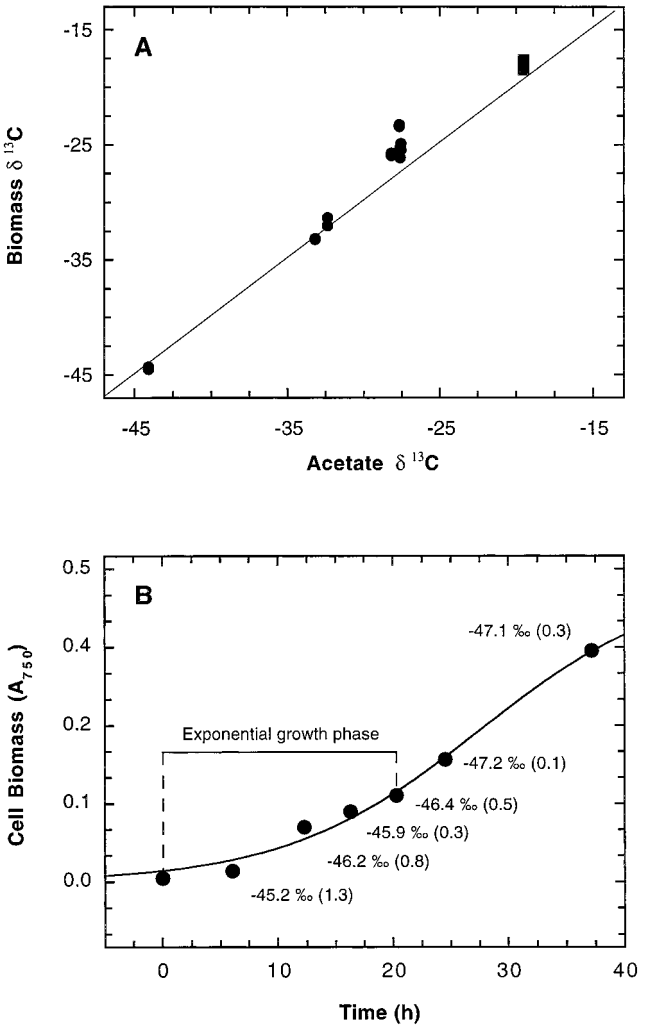

Figure 1. Carbon isotope composition ($\delta^{13}C$) of cell biomass during photoautotrophic and heterotrophic growth of *C. reinhardtii*. A, Heterotrophic growth of *C. reinhardtii* on HSHA containing 29.4 mM acetate of varying $\delta^{13}C$. Cells of the non-photosynthetic mutant strain CC-744 were grown in dim light (5–25 μmol m^{-2} s^{-1}) on HSHA plates (●) or 250-mL HSHA shake flask cultures (■) formulated with sodium acetates differing in natural abundance of ^{13}C. The stable carbon isotope compositions of the source acetates and lyophilized biomass were determined as described in the text. B, Photoautotrophic growth of wild-type *C. reinhardtii* in 250-mL liquid cultures bubbled with 5% CO_2 under moderate light (350 μmol m^{-2} s^{-1}) at 25°C. Samples were harvested at the indicated times for hemocytometer counts, spectrophotometric determination of biomass concentration (A_{750}), and biomass $\delta^{13}C$ measurement. Each value and data point are the means ± SE (in parentheses) of two independent measurements. Data points were fitted to a logistic growth equation. Cell concentration at the beginning of the experiments was 2×10^5 cells mL^{-1} (A_{750} = 0.01).

cultures supplemented with 5% CO_2 at three irradiance levels (200, 350, and 600 μmol m^{-2} s^{-1}) resulted in an average isotopic discrimination relative to the source (Δ) of 24.6‰ ± 0.3‰, which in agreement with earlier data (Sharkey and Berry, 1985). The much more negative biomass $\delta^{13}C$ values in Figure 1B are due to the use of CO_2 sources with different isotopic compositions. The Δ with respect to source CO_2 in cells grown autotrophically on agar plates with 5% CO_2 was 21.9‰ (19.9‰ to 22.6‰ in four separate experiments with different genotypes). In cells of wild-type grown autotrophically on agar plates in air, an even lower discrimination was observed (Δ = 6.3‰ to 10.7‰). Evidently, CO_2 limitations in the wet cell mass on the agar surface and/or the CO_2 concentrating mechanism were responsible for the smaller discriminations. As there was no detectable change in discrimination during heterotrophic growth on HSHA plates or liquid HSHA medium (Fig. 1A), acetate diffusion problems on the agar plates can be ruled out.

Estimation of Carbon Acquisition during Mixotrophic Growth of Wild-Type *C. reinhardtii* and Mutants with Impaired PSII Function

Isotopic composition of mixotrophically grown wild-type *C. reinhardtii* was determined using HSHA (29.4 mM acetate, $\delta^{13}C$ = −27‰ to −44‰) in liquid cultures bubbled with 5% CO_2 ($\delta^{13}C$ = −19.5‰) or on agar plates exposed to ambient CO_2 in air ($\delta^{13}C$ = −8‰). The photosynthetic fraction of carbon biomass (see equation) was calculated from these values, and the baseline heterotrophic and photoautotrophic isotopic composition. Consistent with the inhibitory effects of acetate on photosynthesis (Table I), marked reductions were observed in the fraction of biomass carbon assimilated photosynthetically (Table II). In HSHA liquid medium under saturating light and CO_2, the photosynthetic fraction did not exceed 55%. On plates exposed to air in high light, this fraction was only 23%. Strikingly, wild-type cells grown on HSHA plates exposed to air in moderate or low light showed little or no detectable autotrophic carbon assimilation.

TAP medium (17.5 mM acetate) is commonly used to grow wild-type and mutant strains of *C. reinhardtii* for photosynthetic and molecular analysis (Rochaix et al., 1998). The relative photosynthetic fraction of carbon metabolism in the wild type and in mutations affecting chloroplast protein synthesis and PSII function was determined using TAP medium supplemented with ^{13}C to make the $\delta^{13}C$ acetate much more positive than air or the 5% CO_2 source. The biomass of wild-type cells grown mixotrophically in liquid cultures of TAP medium ($\delta^{13}C$ = approximately +95‰ to +99‰) bubbled with 5% CO_2 ($\delta^{13}C$ =

Table II. *Comparison of the photosynthetic fraction of carbon biomass in wild-type C. reinhardtii grown mixotrophically in HSHA (29.4 mM acetate) liquid medium bubbled with 5% CO_2 and on agar plates exposed to air*

Growth Medium	CO_2 Level	Irradiance	Photosynthetic Fraction of Carbon Biomass
		μmol m^{-2} s^{-1}	*%*
HSHA liquid	5%	600	54
HSHA plates	Air	600	23
		350	9
		<25	0→3

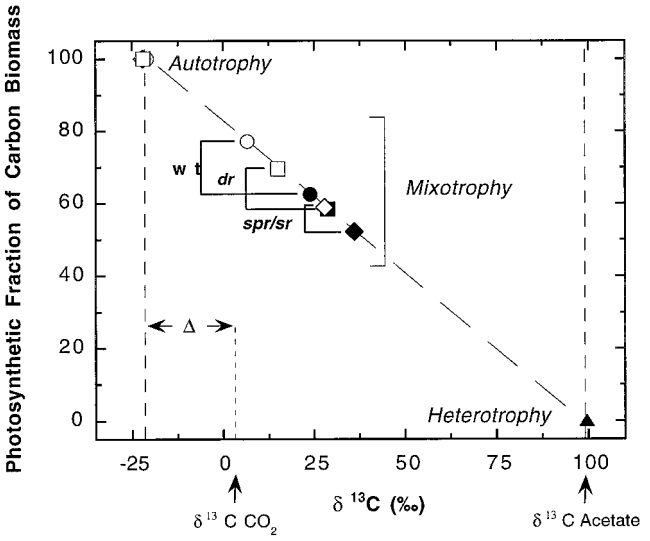

Figure 2. Photosynthetic fraction of carbon biomass from wild-type and mutant *C. reinhardtii* cells grown on ^{13}C-TAP (17.5 mM acetate) in liquid culture. Cells of wild type (circles), *spr/sr* (diamonds), and *dr* (squares) were grown autotrophically to the early-/mid-exponential phase in HS bubbled with 5% CO_2 ($\delta^{13}C$ = +2.9‰), heterotrophically in the dark on TAP supplemented with ^{13}C ($\delta^{13}C$ = +99‰; ▲), and mixotrophically on ^{13}C TAP bubbled with 5% CO_2 at two irradiance levels, 350 μmol m^{-2} s^{-1} (closed symbols) and 600 μmol m^{-2} s^{-1} (open symbols). The fraction of carbon biomass resulting from photosynthetic carbon reduction was calculated as described in the text from the average photoautotrophic, heterotrophic, and mixotrophic biomass $\delta^{13}C$ values.

+2.9‰) showed a photosynthetic fraction of only 78% under saturating irradiance and this declined to 62% at subsaturating irradiance. Thus, even under the optimal light and CO_2 conditions, nearly one quarter of the carbon in the wild type was derived heterotrophically when the cells were provided 17.5 mM acetate and 5% CO_2 as alternative carbon sources. As expected, $\delta^{13}C$ values for the *dr* mutant, with slower PSII electron transfer, showed a lower photosynthetic fraction compared with wild type grown mixotrophically under identical moderate and high light conditions (Fig. 2). The *spr/sr* mutant, which has defects in chloroplast protein synthesis, was even more dependent on heterotrophically assimilated carbon during mixotrophic growth. Reductions in autotrophic competence of the two mutants under high light compared with the wild type correlate well with their impaired light-saturated photosynthetic rates and growth rates (Heifetz et al., 1992, 1997).

Role of the Alternative Oxidase during Mixotrophic and Autotrophic Growth

We established baseline isotopic signatures for respiratory O_2 exchange via the cytochrome oxidase and alternative (cyanide insensitive oxidase) pathways during mixotrophic and photoautotrophic growth of wild-type cells to determine if partitioning between the two respiratory pathways is influenced by acetate. For end point determinations of discrimination due to only the alternative or cytochrome oxidases, wild-type cells were pretreated for 15 min with KCN or the alternative oxidase inhibitor propyl gallate. Alternatively, photoautotrophically grown cells of the *dk-97* mutant lacking cytochrome oxidase activity (Wiseman et al., 1977; Husic and Tolbert, 1987) were used to assess discrimination due to the alternative oxidase pathway. The oxygen isotope discrimination in photoautotrophically grown wild-type cells in minimal medium (Δ = 18.8‰) reveals little engagement of the alternative pathway (Table III), which is in agreement with previous work (Weger et al., 1990b). Respiratory discrimination of wild-type cells grown in the presence of acetate and 5% CO_2 (20.8‰) was not significantly affected by propyl gallate treatment (Table III). This demonstrates that the alternative oxidase was not engaged in the presence or absence of acetate under these conditions. These results, together with the lack of increased dark respiration in mixotrophically grown cells, indicate that the effects of acetate are on photosynthesis rather than on respiration.

DISCUSSION

Our results demonstrate that growth of wild-type *C. reinhardtii* in the presence of 3.7 to 29.4 mM acetate in saturating light and CO_2 inhibits photosynthesis, as measured by the maximum rates of net O_2 evolution and ^{14}C fixation. However, neither dark respiration nor engagement of the alternative oxidase pathway were affected. Fett and Coleman (1994) reported that acetate stimulated res-

Table III. *Discrimination against $^{18}O_2$ during dark respiration by wild-type C. reinhardtii cells grown to the mid-exponential phase at 600 μmol m^{-2} s^{-1} irradiance in liquid cultures bubbled with 5% CO_2*

KCN results in discrimination due solely to O_2 consumption via the alternative oxidase pathway. Propyl gallate results in discrimination due solely to O_2 consumption via the cytochrome oxidase pathway.

Genotype	Medium	Inhibitor	Discrimination	*n*
			% ± SE	
dk-97	HS	None	24.3 ± 0.5	3
Wild type	HS	None	18.8 ± 0.4	10
Wild type	HS	1 mM KCN	24.2 ± 1.2	5
Wild type	TAP	None	20.8 ± 0.8	6
Wild type	TAP	l mM KCN	24.2 ± 1.9	5
Wild type	TAP	500 μM Propyl gallate	21.1 ± 0.9	4

piration in cells grown mixotrophically in air, and Endo and Asada (1996) demonstrated a similar response immediately upon addition of acetate to autotrophically grown cells. Growth rates in our experiments were unaffected by the large reduction in photosynthesis in the presence of acetate (Table I). Moreover, analysis of stable carbon isotope composition of biomass from mixotrophically grown cells revealed a marked shift from autotrophic to heterotrophic carbon metabolism in response to both environmental and genetic manipulation of *C. reinhardtii*. The stable isotope data (Table II) indicate that carbon derived from acetate in the light can substitute for up to 50% of photoautotrophically acquired carbon in liquid cultures under the saturating light and CO_2 conditions optimal for photosynthetic growth of *C. reinhardtii* (Heifetz et al., 1997). At subsaturating irradiance and CO_2 levels in the presence of specific mutations reducing photosynthetic performance, further decreases in the contribution of photosynthetic carbon assimilation were observed under mixotrophic growth conditions.

Although one might expect that the addition of a reduced carbon source would lower the proportion of biomass carbon derived from photosynthesis, the notion that acetate metabolism in saturating light and CO_2 can quantitatively substitute for photosynthetic carbon assimilation to drive growth in *C. reinhardtii* is probably overly simplistic. There is undoubtedly a dynamic relationship between acetate metabolism and photosynthesis that involves both mitochondria and chloroplasts. Consistent with other treatments that deplete cell ATP, Gans and Rebéillé (1990) found that the addition of acetate to autotrophically grown *C. reinhardtii* decreased PSII fluorescence and promoted a transition from state I to state II, presumably with attendant adjustment of the antenna architecture of the photosynthetic apparatus (Bulté et al., 1990). These observations were confirmed and extended by Endo and Asada (1996), who showed that the addition of acetate produced transient non-photochemical quenching in the light, which was sustained in the dark and associated with a reduction in PSII efficiency. Whether these primary events, thought to be mediated by chlororespiration (Bennoun, 1998), account for the long-term decline in photosynthetic O_2 evolution and carbon assimilation observed here remains to be assessed. Greater inhibition of photosynthesis by acetate at lower light intensities (Table II; Fig. 2) would be consistent with such a reduction in PSII efficiency, but this was not reflected in our dark-adapted measurements of F_v/F_m (Table I).

The first step in acetate utilization is the ATP-dependent production of acetyl coenzyme A. Therefore, in mixotrophic growth under limiting light, ATP demand for acetate assimilation may itself limit photosynthetic carbon reduction. These effects may be exacerbated if CO_2 is limited due to the induction of the carbon concentrating mechanism (Spalding, 1998). Acetate may also exert inhibitory effects on metabolism, as concentrations above 6.7 mM were reported to inhibit heterotrophic growth of wild-type *C. reinhardtii* (Chen and Johns, 1994). In the absence of acetate, reduced photosynthesis in several *C. reinhardtii* mutants with impaired D1 function (Fšrster et al., 1997; Lardans et al., 1998) or resistance to very high light (Förster et al., 1999) did not directly affect growth rate. These observations suggest that metabolic variables other than photosynthetic CO_2 fixation may sometimes limit growth.

The mechanisms underlying the effects of acetate on photosynthesis in our long-term growth experiments may also involve changes in gene expression. In plants and algae, carbon metabolites (including acetate) are known to down-regulate the expression of nuclear genes encoding chloroplast proteins involved in photosynthesis and in non-photosynthetic carbon metabolism (Kindle, 1987; Sheen, 1990, 1994). At the transcriptional level, acetate is a potent repressor of synthesis of enzymes involved in photosynthetic carbon reduction, as well as an inducer of the glyoxylate cycle-specific enzymes malate synthetase (Neilson and Lewin, 1974) and isocitrate lyase (Martinez-Rívas and Vega, 1993). Thus, transcriptional and translational regulation of both nuclear and chloroplast genes encoding photosynthetic components and enzymes involved in acetate metabolism would be expected to respond dynamically to the presence of acetate. These molecular processes, as well as the physiological events they influence, should therefore be compared in both mixotrophically and photoautotrophically grown cells.

In summary, we show that both photosynthetic incorporation of inorganic carbon and the maximum rate of O_2 evolution in *C. reinhardtii* can be significantly diminished by growth in the presence of acetate. Under some circumstances (limiting light, high acetate concentrations, growth on solid medium in air) photosynthetic carbon gain is virtually abolished. In studies involving mutants of *C. reinhardtii* with partial photosynthetic defects that do not cause obligate heterotrophy, very different interpretations of their metabolic consequences might be obtained depending on the presence or absence of acetate. Consequently, interpretation of the effects of environmental or other manipulations may be confounded by acetate-induced impairment of photosynthetic performance.

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