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Characterization of an Electron Transport Pathway Associated with Glucose and Fructose Respiration in the Intact Chloroplasts of *Chlamydomonas reinhardtii* and Spinach[1]

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ABSTRACT

The role of an electron transport pathway associated with aerobic carbohydrate degradation in isolated, intact chloroplasts was evaluated. This was accomplished by monitoring the evolution of $^{14}CO_2$ from darkened spinach (*Spinacia oleracea*) and *Chlamydomonas reinhardtii* chloroplasts externally supplied with [^{14}C]fructose and [^{14}C]glucose, respectively, in the presence of nitrite, oxaloacetate, and conventional electron transport inhibitors. Addition of nitrite or oxaloacetate increased the release of $^{14}CO_2$, but it was shown that O_2 continued to function as a terminal electron acceptor. $^{14}CO_2$ evolution was inhibited up to 30 and 15% in *Chlamydomonas* and spinach, respectively, by 50 μM rotenone and by amytal, but at 500- to 1000-fold higher concentrations, indicating the involvement of a reduced nicotinamide adenine dinucleotide phosphate-plastoquinone oxidoreductase. $^{14}CO_2$ release from the spinach chloroplast was inhibited 80% by 25 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone. $^{14}CO_2$ release was sensitive to propylgallate, exhibiting approximately 50% inhibition in *Chlamydomonas* and in spinach chloroplasts of 100 and 250 μM concentrations, respectively. These concentrations were 20- to 50-fold lower than the concentrations of salicylhydroxamic acid (SHAM) required to produce an equivalent sensitivity. Antimycin A (100 μM) inhibited approximately 80 to 90% of $^{14}CO_2$ release from both types of chloroplast. At 75 μM, sodium azide inhibited $^{14}CO_2$ evolution about 50% in *Chlamydomonas* and 30% in spinach. Sodium azide (100 mM) combined with antimycin A (100 μM) inhibited $^{14}CO_2$ evolution more than 90%. $^{14}CO_2$ release was unaffected by uncouplers. These results are interpreted as evidence for a respiratory electron transport pathway functioning in the darkened, isolated chloroplast. Chloroplast respiration defined as $^{14}CO_2$ release from externally supplied [1-^{14}C]glucose can account for at least 10% of the total respiratory capacity (endogenous release of CO_2) of the *Chlamydomonas reinhardtii* cell.

Sugars added to and starch within the chloroplast are degraded through a series of reactions resulting initially in the formation of glucose and fructose phosphates (14, 25, 31). The conversion of these hexose phosphates to CO_2, dihydroxyacetone-P, and 3-phosphoglycerate, the major species exported from the chloroplast, is facilitated by the reactions either of glycolysis (Embden-Meyerhof Pathway) or of the oxidative pentose-P pathway (Lipmann-Dickens Pathway) coupled to glycolysis (1, 9, 32). Inasmuch as both pathways involve oxidative steps, the operation of these multienzymic pathways requires a constant supply of oxidized pyridine nucleotide (hydrogen cycle).

The balancing of pyridine nucleotides in the chloroplast per se is essential for dissimilation of carbohydrates due to the limited movement of nicotinamide nucleotides across the chloroplast's envelope. In complete gylcolysis, acetaldehyde or pyruvate, which serves as the physiological hydrogen acceptor, is derived from glycerate-3-P. The chloroplast seems to lack the lower (glycerate-3-P to pyruvate) reactions of glycolysis (16). There are no reports that the necessary dismutating enzymes, alcohol and lactate dehydrogenases, are localized within the chloroplast.

Recent studies on thylakoidal fractions of *Chlamydomonas reinhardii* revealed the presence of a NAD(P)H-PQ[3] oxidoreductase similar to that associated with NADH oxidation in mitochondria (13). An uncharacterized NADPH oxidizing system in *C. reinhardii* thylakoids was reported earlier (6) and was interpreted to function in the evolution of H_2 from carbohydrates. The oxidation of PQ by O_2 has been shown in other studies with *Chamydomonas* (7, 22, 26), indicating the components necessary for a respiratory pathway in the algal chloroplast. The O_2-dependent evolution of $^{14}CO_2$ from darkened spinach chloroplasts externally supplied with [^{14}C]glucose is consistent also with a respiratory pathway in the higher plant organelle (1).

In the present study, we evaluate the possible role of an electron transport pathway associated with aerobic carbohydrate degradation in isolated, intact *C. reinhardtii* and spinach chloroplasts. This was accomplished by monitoring the release of $^{14}CO_2$ from the darkened chloroplasts supplied with [^{14}C]glucose and [^{14}C]fructose in the presence of OAA, nitrite, and conventional electron transport inhibitors.

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[3] Abbreviations: PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FCCP, carbonyl cyanide-*p*-trifluoro-methoxyphenylhyddrazone; SHAM, salicylhydroxamic acid; OAA, oxaloacetate.

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MATERIALS AND METHODS

Plant Material and Chloroplast Isolation

Spinach (*Spinacia oleracea* L.) var Longstanding Bloomsdale was grown and used to prepare intact chloroplasts as described earlier (19). *Chlamydomonas reinhardtii* 137C (+) and 137C (−) and the mutant strain F-60 (obtained from R.K. Togasaki, Indiana University, Bloomington, IN) were grown under fluorescent lights on an acetate-supplemented medium as described previously (36). The procedure for isolating chloroplasts from F-60 followed those of Klein et al. (17).

$^{14}CO_2$ Measurements

Rates of CO_2 evolution were determined by adding intact chloroplasts of F-60 to a reaction mixture containing 50 mM Tris-HC (pH 8.2), 120 mM mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM MgATP, and [U-^{14}C]glucose. In the case of spinach, intact chloroplasts were added to a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM MgATP, and [U-^{14}C]fructose.

The reaction mixtures were incubated at 25°C for 10 min in the main compartments of darkened 15-mL Warburg flasks carrying the chloroplast suspension in a sidearm and 0.3 mL of 2 M triethanolamine in the center well. The vessels were sealed with serum stoppers and the reactions were initiated by tipping in the chloroplasts from the sidearm to bring the final reaction mixture volume to 1.0 mL. If anaerobic conditions were desired, the Warburg flasks were flushed by five cycles of vacuum with a water aspirator and refilled with O_2-free N_2 and then flushed continuously with N_2 for 10 min prior to the addition of the chloroplast suspension into the reaction mixture. Following termination of the reaction by addition of perchloric acid to a final concentration of 5% (v/v), the flasks were shaken slowly for an additional hour to allow the released $^{14}CO_2$ to be absorbed totally by the triethanolamine. The triethanolamine was transferred into a scintillation vial containing SML Biodegradable counting scintillant (Amersham). Radioactivity was determined in a Beckman L5–150 liquid scintillation counter.

Chl Determination

Chl was assayed by the method of Arnon (3).

Reagents

Radioactive sugars were purchased from ICN Radiochemicals and DuPont NEN. Unlabeled materials were purchased from Sigma. Tank gases were supplied by Matheson.

Amytal rotenone, DBMIB, antimycin A, and propylgallate were dissolved in DMSO. SHAM and sodium azide were dissolved in water. The final concentration of 0.5% DMSO in the reaction mixture inhibited the assay less than 5%.

RESULTS AND DISCUSSION

Purity of the Chloroplast Preparations

The $^{14}CO_2$ assay takes advantage of the fact that the decarboxylative reaction catalyzed by NADP-dependent gluconate-6-P dehydrogenase of the oxidative pentose-P pathway appears to be the sole enzyme responsible for the release of CO_2 during glucose degradation in isolated *Chlamydomonas* (9) and spinach (1, 32) chloroplasts. We supply the evidence that $^{14}CO_2$ evolution was due to intact chloroplasts and not to cytoplasmic or bacterial contamination. First, chloroplast integrity was required for $^{14}CO_2$ production from [^{14}C]glucose and [^{14}C]fructose. $^{14}CO_2$ formation from [^{14}C]sugar by the lysed chloroplasts was less than 10% of that by the intact chloroplasts. Second, fluoroacetate and malonate, which block cellular respiration by inhibiting mitochondrial metabolism, did not diminish $^{14}CO_2$ production from [^{14}C]sugar. Third, intermediates of the citric acid cycle (10), including α-ketoglutarate, fumarate, and succinate, which are known to penetrate the chloroplast, and gluconate-6-P, which does not, did not affect $^{14}CO_2$ generation. Fourth, ATP, glycolate-2-P and P-*enol*pyruvate, which do not penetrate intact cells, are effectors of [^{14}C]sugar respiration in the darkened chloroplasts (1, 9, 32). Fifth, light and DTT, which would be expected to decrease sugar oxidation in the chloroplasts due to inhibition of glucose-6-P dehydrogenase (2), essentially eliminated $^{14}CO_2$ release. This inhibition was not due to photosynthetic assimilation of the respired $^{14}CO_2$ because the F-60 mutant lacks ribulose-5-P kinase (24), and DL-glyceraldehyde (33) was included in the reaction mixture containing the spinach chloroplasts.

Fructose and Glucose Respiration

Spinach and *Chlamydomonas* released $^{14}CO_2$ when incubated with [U-^{14}C]fructose and [U-^{14}C]glucose, respectively (Table I). In an earlier communication on spinach chloroplasts (1), glucose was utilized as the respirable substrate. In this study, fructose was the externally added sugar, because we now report that $^{14}CO_2$ is evolved from fructose at a rate roughly 3 to 4 times that of glucose. Consistent with this result is the report of Schnarrenberger (28) that indicates that spinach leaf fructokinase and to a lesser extent glucokinase are localized in the isolated chloroplast. Miernyk and Dennis (23) observed earlier this enzymic compartmentation for plastids in developing castor bean endosperm tissue. In contrast, fructose is respired weakly by the F-60 chloroplast (data not shown).

Under anaerobic conditions, which resulted in a decreased evolution of $^{14}CO_2$, nitrite and OAA were used as "alternate" terminal acceptors for spinach and *Chlamydomonas*, respectively. An increase in the aerobic rate of $^{14}CO_2$ release when the chloroplasts were supplemented with nitrite or OAA indicates a similar function even in the presence of O_2. Thus, the respiratory process was not limited by labeled substrate. It is worth noting that OAA functioned poorly in the spinach chloroplast and nitrite was not suitable for the algal chloroplast because F-60 lacks nitrite reductase.

The following sequence of events, as first developed by Kow et al. (18) with a reconstituted spinach chloroplast

Table I. *Effect of OAA and Nitrite on Aerobic and Anaerobic Respiration in Chlamydomonas and Spinach Chloroplasts*

Chlamydomonas: Included in the reaction mixture described in "Materials and Methods" were 525 μM [U-^{14}C]glucose (3.3 mCi/mmol) and a Chl content of 24.8 μg. Spinach: Included in the reaction mixture described in "Materials and Methods" were 500 μM [U-^{14}C]fructose (2 mCi/mmol) and a Chl content of 50 μg. The reactions were carried out in the dark at 25°C and were terminated after 30 min with perchloric acid.

	Chlamydomonas		Spinach	
	OAA	CO_2 evolved	Nitrite	CO_2 evolved
	μM	*nmol/mg Chl·h*	μM	*nmol/mg Chl·h*
Air	0	178	0	34
	0.5	295	1	39
	1.0	344	5	46
	2.5	249	10	54
	5.0	335	100	96
N_2	0	31	0	5
	0.5	70	1	15
	1.0	88	5	27
	2.5	209	10	33
	5.0	199	100	52

preparation, can serve as an explanatory basis for the results recorded in Table I.

$$\text{Glucose-6-P} + \text{NADP} \rightarrow \text{Gluconate-6-P} + \text{NADPH} \quad (1)$$

$$\text{NADPH} + 2\ \text{Fd}_o \rightarrow 2\ \text{Fd}_r + \text{NADP} \quad (2)$$

$$2\ \text{Fd}_r + \text{O}_2 \rightarrow 2\ \text{Fd}_o + \text{H}_2\text{O}_2 \quad (3)$$

Anaerobicity prohibits the reaction expressed in Equation 3 from taking place, resulting in an accumulation of NADPH and Fd_r. The operation of a malate/OAA shuttle coupled to malate dehydrogenase in the algal chloroplast can account for the direct oxidation of NADPH generated in the oxidative pentose-P pathway. Additionally, Fd_r could act as the electron donor for the reduction of nitrite to NH_3 via nitrite reductase and result in a restoration of $^{14}CO_2$ release in the spinach chloroplast.

Effect of Inhibitors on CO_2 Release

Another potential pathway for balancing the chloroplastic hydrogen cycle with molecular O_2 is an integrated respiratory chain functioning in the darkened organelle. The use of inhibitors has been one conventional approach to the elucidation of complex electron transport chains. To this end, a variety of representative respiratory inhibitors, including rotenone, amytal, DBMIB, antimycin A, NaN_3, SHAM, and propylgallate, were tested for their ability to affect the release of $^{14}CO_2$ from chloroplasts externally supplied [U-^{14}C]sugar.

Studies on thylakoidal functions of *C. reinhardtii* have revealed the presence of a NAD(P)H-PQ oxidoreductase similar to that associated with NADH oxidation in mitochondria (13). A concentration of 100 μM rotenone was needed to inhibit the enzyme to 50%. Under the conditions of the oxyhydrogen reaction, CO_2 fixation in whole *Chlamydomonas* cells was also inhibited approximately 90% by 150 μM rotenone, but because of the presence of 1% O_2 in the reaction mixture, this inhibition could be attributed to an effect on either thylakoidal PQ oxidoreductase or mitochondrial ubiquinone oxidoreductase (22). Rotenone at the highest concentration tested (50 μM) inhibited $^{14}CO_2$ evolution in *Chlamydomonas* and spinach chloroplasts (Fig. 1) 30 and 15%, respectively, whereas amytal, which mimics most of the known features of rotenone, was slightly more effective, but at 500- to 1,000-fold higher concentrations (Fig. 2). Concentrations of rotenone beyond 50 μM were found to affect chloroplast integrity.

DBMIB, a powerful inhibitor of photosynthetic (34) and mitochondrial (30) electron transport, acts as an antagonist to PQ. Of all the poisons tested against the oxyhydrogen in *Chlamydomonas* reaction (22), DBMIB was the most potent, with 100% inhibition at a concentration of 5 μM. Likewise, with $^{14}CO_2$ evolution in the spinach chloroplast (Fig. 3), DBMIB was the strongest inhibitor tested, with an 80% inhibition at 25 μM. Noteworthy is the 50% increase in $^{14}CO_2$ evolution at 5 μM DBMIB. We have interpreted this stimulatory effect of DBMIB as a competitive relation between PQ and DBMIB for electrons from the NAD(P)H-donating oxidoreductase followed by an autooxidation of reduced DBMIB. Thus, DBMIB accepts and shunts electrons directly to O_2. Because autooxidation is a slow reaction (21), reduced DBMIB, the inhibitor, accumulates at the higher concentrations of the PQ antagonist, resulting eventually in an inhibition of CO_2 release.

CO_2 evolution from both types of chloroplast was sensitive to SHAM (Fig. 4) and to propylgallate (Fig. 5), well-established inhibitors of the cyanide-insensitive branch of mitochondrial-mediated respiration (20). The concentration of propylgallate required to obtain roughly 50% inhibition was 100 μM in *Chlamydomonas* and 250 μM in spinach. These

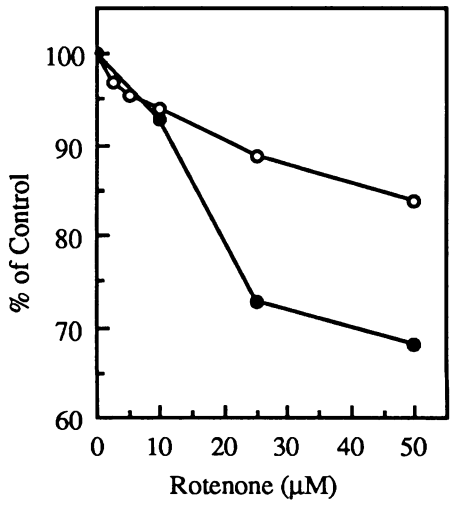

Figure 1. Effect of rotenone on $^{14}CO_2$ release from spinach and *Chlamydomonas* chloroplasts. Control rates with spinach (90 μg Chl/mL, 500 μM [U-^{14}C]fructose, 2 mCi/mmol) (○) and *Chlamydomonas* (36 μg Chl/mL, 550 μM [U^{14}]glucose, 2.9 mCi/mmol) (●) were 44 and 181 nmol/mg Chl·h, respectively.

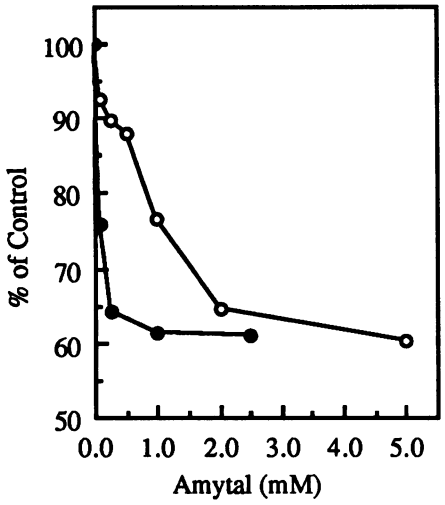

Figure 2. Effect of amytal on $^{14}CO_2$ release from spinach and *Chlamydomonas* chloroplasts. Control rates with spinach (80 μg Chl/mL, 500 μM [U-^{14}C]fructose, 2 mCi/mmol) (O) and *Chlamydomonas* (25 μg Chl/mL, 550 μM [U-^{14}C]glucose, 2.9 mCi/mmol) (●) were 40 and 180 nmol/mg Chl·h, respectively.

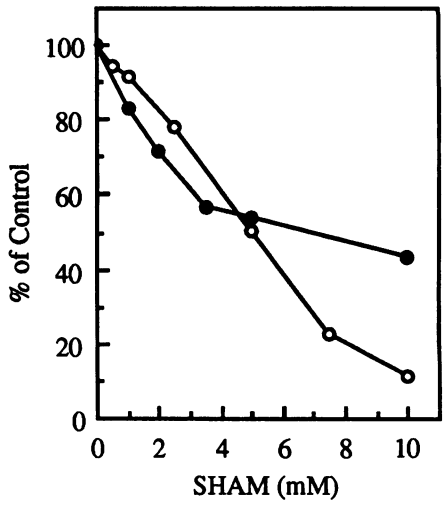

Figure 4. Effect of SHAM on $^{14}CO_2$ release from spinach and *Chlamydomonas* chloroplasts. Control rates with spinach (77 μg Chl/mL, 500 μM [U-^{14}C]fructose, 2 mCi/mmol) (O) and *Chlamydomonas* (20 μg Chl/mL, 550 μM [U-^{14}C]glucose, 2.9 mCi/mmol) (●) were 44 and 216 nmol/mg Chl·h, respectively.

values are 20- to 50-fold lower than the concentration of SHAM required to inhibit the process in the two chloroplasts.

On the basis of studies with plant mitochondrial respiration (20, 27, 29) and with the oxyhydrogen reaction in *Chlamydomonas* cells (22), we speculate that SHAM and propylgallate share a common inhibitory site in CO_2 release mediated by the intact algal and higher plant chloroplasts. Given the significantly lower (about 20-fold) concentration of propylgallate required to inhibit chloroplast respiration, propylgallate is a useful alternative to SHAM, as first demonstrated by Siedow and Girvin (29) in their investigation with mung bean mitochondria.

Antimycin A was found to be a potent inhibitor of CO_2 evolution, with the algal chloroplast exhibiting greater sensitivity (Fig. 6). At 100 μM antimycin A, the highest concentration tested, the process was inhibited about 90 and 80% in *Chlamydomonas* and spinach, respectively. The inhibition by antimycin A, which is known to affect a *b*-type Cyt associated with mitochondrial electron transport systems, may indicate a function for other components of the thylakoidal chain in association with the PQ oxidoreductase. To this end, the sensitivity of CO_2 evolution to NaN_3 was examined. At a concentration of 75 mM, NaN_3 inhibited CO_2

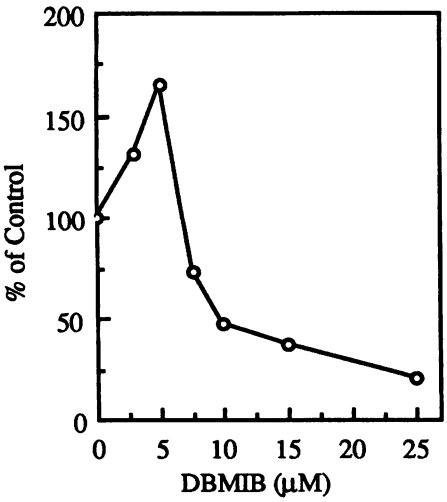

Figure 3. DBMIB inhibition of $^{14}CO_2$ release from spinach chloroplasts. Control rate was 49 nmol/mg Chl·h (77 μg Chl/mL, 500 μM [U-^{14}C]fructose, 2.9 mCi/mmol).

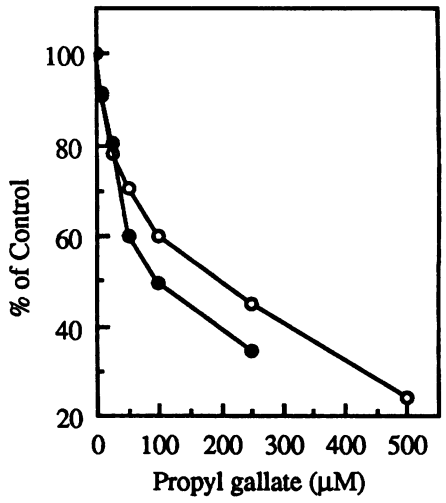

Figure 5. Effect of propylgallate on $^{14}CO_2$ release from spinach and *Chlamydomonas* chloroplasts. Control rates with spinach (78 μg Chl/mL, 500 μM [U-^{14}C]fructose, 2 mCi/mmol) (O) and *Chlamydomonas* (50 μg Chl/mL, 550 μM [U-^{14}C]glucose, 2.9 mCi/mmol) (●) were 45 and 216 nmol/mg Chl·h, respectively.

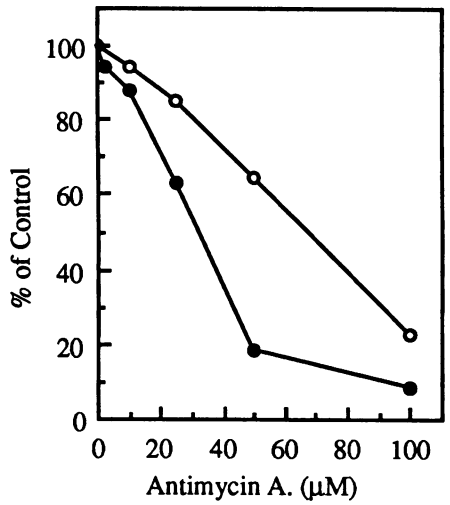

Figure 6. Antimycin A inhibition of $^{14}CO_2$ release from spinach and *Chlamydomonas* chloroplasts. Control rates with spinach (65 μg/mL, 500 μM [U-^{14}C]fructose, 2 mCi/mmol) (○) and *Chlamydomonas* (19 μg Chl/mL, 550 μM [U-^{14}C]glucose, 2.9 mCi/mmol) (●) were 59 and 170 nmol/mg Chl·h, respectively.

release about 50% in *Chlamydomonas* and 30% in spinach (Fig. 7). Azide was used instead of the customary cyanide in order to avoid the formation of the cyano- addition product with glucose and fructose (20). On the other hand, azide is less active than cyanide in blocking mitochondrial electron flow (20).

As demonstrated by Werdan et al. (35), the uncharged HN_3 molecule penetrates the chloroplast envelope, and once inside the stroma, ionizes to release a proton and the charged N_3 ion, which is the inhibitor. When this occurs across the chloroplast envelope, the stromal pH falls. Inasmuch as CO_2 evolution is sensitive to pH (9) and the optimum pH value for CO_2 release by each chloroplast was used, inhibition at concentrations of NaN_3 higher than 100 mM might reflect a decrease in stromal pH in addition to a selective inhibitory site.

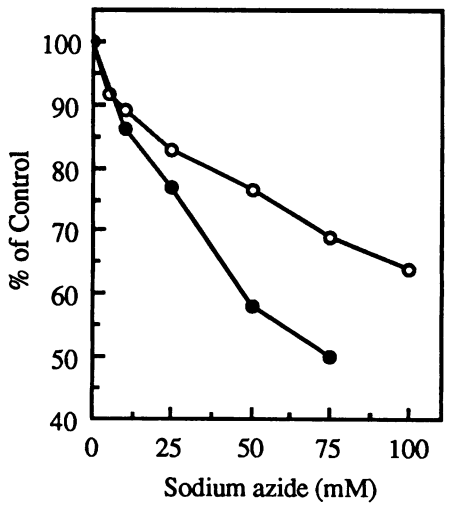

Figure 7. Effect of sodium azide on $^{14}CO_2$ release from spinach and *Chlamydomonas* chloroplasts. Control rates with spinach (90 μg Chl/mL, 500 μM [U-^{14}C]fructose, 2 mCi/mmol) (○) and *Chlamydomonas* (22 μg Chl/mL, 550 μM [U-^{14}C]glucose, 2.9 mCi/mmol) (●) were 46 and 225 nmol/mg Chl·h, respectively.

The uncouplers FCCP (up to 1.0 μM) and NH_4Cl (up to 10 mM) did not influence CO_2 evolution (data not shown). Furthermore, the inhibitory properties of amytal, antimycin A, propylgallate, and sodium azide were unaffected by the addition of the uncouplers to the reaction medium. This ineffectiveness of the uncouplers may indicate that the respiratory process in the intact chloroplast was not coupled to ATP synthesis or that ADP was present in excess. On the other hand, substrate was not a limiting factor because nitrite and OAA increased the rate of CO_2 release (Table I).

The results recorded in Table I indicate the inadequacy of the O_2-dependent pathway in sustaining the maximum capacity of pyridine nucleotide turnover in both types of chloroplast. We attempted to determine whether O_2 continues to contribute to pyridine nucleotide turnover in the spinach chloroplast in the presence of an alternate electron acceptor by measuring the effect of three respiratory inhibitors on CO_2 release with nitrite in the reaction medium (Table II). It is quite clear that inhibition of CO_2 release by amytal, antimycin A, and propylgallate is sustained, indicating that nitrite does not divert electron flow totally from the O_2-dependent pathway.

CONCLUSION

The results of our inhibitor studies with the intact chloroplasts of *C. reinhardtii* and spinach indicate that the respiratory electron transport pathway functioning in the darkened chloroplasts has many properties in common with the electron transport pathway of mitochondria. Our results with rotenone and amytal (Figs. 1 and 2), inhibitors of PQ reduction, and with DBMIB (Fig. 3), an inhibitor of PQ oxidation, indicate that electron flow through the PQ pool catalyzed by NAD(P)H-PQ oxidoreductase is an important aspect of the

Table II. *Effect of Nitrite and Respiratory Inhibitors on $^{14}CO_2$ Release in Spinach Chloroplasts*

Included in the mixture described in "Materials and Methods" were 500 μM [U-^{14}C]fructose (2.0 mCi/mmol), a Chl content of 52 μg, and where indicated, 100 μM KNO_2, 100 μM antimycin A, 500 μM propylgallate, or 5 mM amytal. Final concentration of DMSO in the reaction mixtures was 0.5%. The reactions were carried out in the dark at 25°C and were terminated after 30 min with perchloric acid.

Additions	CO_2 Evolved
	nmol/mg Chl·h
Control	36
KNO_2	113
Antimycin A	25
Antimycin A, KNO_2	68
Propylgallate	14
Propylgallate, KNO_2	50
Amytal	25
Amytal, KNO_2	50

respiratory pathway. The inhibition of CO_2 release by antimycin A (Fig. 6), and NaN_3 (Fig. 7), which are known to affect a *b*-type Cyt and a *c*-type Cyt, respectively, in plant mitochondrial respiration, may indicate a function for other components of the respiratory electron transport chain in association with the PQ-oxidoreductase. We speculate that the PQ-photooxidative process described by Bennoun (7) may be sensitive to antimycin A and NaN_3.

The inhibition of CO_2 evolution by SHAM (Fig. 4) and propylgallate (Fig. 5) suggests similarity between the chloroplast oxidative pathway and the alternate cyanide-insensitive respiratory pathway of plant mitochondria. It is also possible that they compete with the same PQ pool as DBMIB, albeit at higher concentrations. As shown in Figures 4 and 5, SHAM and propylgallate inhibited CO_2 release at least 75%. Furthermore, titration of CO_2 release from spinach chloroplasts with combinations of sodium azide with SHAM and propylgallate (data not shown), and analysis of the results by the method of Bahr and Bonner (5) did not yield a pattern of inhibition suggestive of a branched electron transport system. Accordingly, we interpret our data to indicate a linear respiratory oxidative pathway in this property resembling the photosynthetic rather than the mitochondrial electron transport chain.

Supportive evidence of a linear chain is our observation that the combination of 100 mM sodium azide and 100 μM antimycin A inhibited CO_2 evolution from spinach chloroplasts approximately 95%. The residual release of CO_2 may be due to the reactions (Eqs. 1, 2, and 3) proposed by Kow et al. (18). Clearly, this sequence, based upon the aerobic oxidation of reduced Fd (Mehler reaction) and demonstrated in a reconstituted spinach chloroplast preparation, appears to be a minor pathway in the intact chloroplast.

It is of interest to estimate the contribution of chloroplast respiration to the respiratory capacity of the whole cell. The endogenous rate of respiration of *C. reinhardtii* cells is about 6 to 8 μmol CO_2/mg Chl·h (12). Removal of the impedance of electron transport by addition of an uncoupler, FCCP, elevated the rate to 28 to 30 μmol CO_2/mg Chl·h. Using [1-^{14}C]glucose as substrate, the maximal rate of CO_2 evolution observed with the F-60 chloroplast was 1.5 μmol/mg Chl·h (8). As noted earlier, this rate was not affected by FCCP. The assay of CO_2 release from the chloroplast supplied externally with a monosaccharide and ATP is not an absolute, but a practical way of knowing the maximal rate of respiration. For instance, in contrast to what occurs in the whole cell, the end product of glycolytically degraded sugar in the isolated chloroplast is not CO_2, but glycerate-3-P. Movement of the substrate, simple sugar, or sugar phosphate from medium to the stromal compartment is probably constraining the assay (9). Furthermore, Chl content is not a natural unit of respiration, and is even less so when the major organelle of cellular respiration does not contain the pigment. Nevertheless, Chl appears to be a reasonable compromise for this purpose. Practical difficulties notwithstanding, chloroplast respiration comprises at least 10 to perhaps 20% of the total respiration of the "resting" algal cell.

The significance of the chloroplast respiratory pathway is not well understood, but its demonstration can clarify a number of reported observations. Cooperation between the oxidative pentose-P pathway and nitrite assimilation can account for the growth of green algae such as *Chlorella* and *Scenedesmus* in the dark, with nitrate as the sole source of nitrogen. Likewise, the chloroplastic pathway may be responsible for the coupling of nitrite reduction to carbohydrate breakdown by barley seedlings in the dark (4). Fifty years ago, Gaffron (11) demonstrated the coupling of CO_2 reduction with the oxyhydrogen reaction in the darkened green algal cell. Compartmentation within the chloroplast of both the photosynthetic carbon reduction cycle and carbohydrate respiration, the latter apparently providing the assimilatory power, is a testable explanation of this phenomenon. Finally, a supply of intermediates of the photosynthetic carbon reduction cycle during the inductive phase of photosynthesis may be an essential contribution of chloroplastic dark metabolism (15).

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