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Energetic Factors Affecting Carbon Dioxide Fixation in Isolated Chloroplasts[1]

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RUDOLF E. SLOVACEK AND GEOFFREY HIND
Biology Department, Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT

Light- and HCO_3^--saturated (10 millimolar) rates of O_2 evolution (120 to 220 micromoles O_2 per milligram chlorophyll per hour), obtained with intact spinach chloroplasts, are decreased up to 3-fold by changes in assay conditions such as omission of catalase from the medium, the use of high (≥1 millimolar) inorganic phosphate, inclusion of NO_2^- as an electron acceptor, or bright illumination at low partial pressures of O_2. These inhibitions may be reversed by addition of uncoupling levels of NH_4Cl or of antimycin concentrations that partially block cyclic electron transfer between cytochrome b_6 and cytochrome *f*. Measurements of the pH gradient across the thylakoid membrane with the fluorescent probe, 9-aminoacridine, indicate that changes in ΔpH are sufficient to account for both the inhibited and restored rates of electron transport. It follows that the rate of HCO_3^--saturated photosynthesis may be restricted by a proton gradient back pressure under these conditions.

The rate of O_2 evolution is also decreased 3-fold when ambient CO_2 (0.63 millimolar HCO_3^- at pH 8.1) is used in place of saturating HCO_3^- and chloroplasts are illuminated aerobically with catalase and a low level (0.25 millimolar) of K_2HPO_4. Only inhibitory effects are observed with additions of antimycin or NH_4Cl. Under these conditions, excessive photophosphorylation or a large pH gradient does not limit the rate of photosynthesis.

Operation of the Calvin cycle during CO_2 fixation requires the production of ATP and NADPH, in the ratio of 3:2 (2), by coupled photosynthetic electron transport. It follows that uncouplers or inhibitors of photophosphorylation, such as NH_4Cl (16) and antimycin (1), should adversely affect the rate of CO_2 fixation.

Early studies (5, 26) provided evidence for this, although in some laboratories the carbon reduction rates could be enhanced by either antimycin (23, 24, 26) or NH_4Cl (7, 14, 20). To reconcile this apparent contradiction it was proposed that photosynthetic carbon reduction is regulated by the activities of a few enzymes within the Calvin cycle and that NH_4Cl or antimycin might indirectly modify these activities (7, 17, 20, 23, 24, 36). Migniac-Maslow and Champigny (17) suggested that increased enzyme activity might result from increased changes in stromal pH and Mg^{2+} ion levels, supported by accelerated electron transport in the presence of antimycin. More specifically, Walker *et al.* (36) proposed that fructose 1,6-bisphosphatase activity is enhanced by antimycin through a direct increase in the level of reduced ferredoxin and operation of the ferredoxin-thioredoxin activator system (38).

The proposed mechanism for acceleration of CO_2 fixation by NH_4Cl (7, 20) involves penetration of NH_3 into the stroma where the uncharged molecule acquires a proton and raises the pH toward the pH optimum of 8.1 for enzymic activity (37). In support of this hypothesis, Heath and Leech (7) have shown that incubation of chloroplasts at pH 7.6 or at 8.1, in the presence of acetate, lowers the rate of HCO_3^--dependent O_2 evolution in a manner which is reversed by NH_4Cl additions. Similarly, Purczeld *et al.* (20) reported that acidification of the stroma with NO_2^- could be reversed by mM levels of NH_4Cl to give an increased rate of CO_2 fixation.

The above mechanisms ascribing increased photosynthesis to elevated enzyme activities resulting from stromal H^+ or Mg^{2+} activity changes are not supported by other experimental observations made with antimycin or NH_4Cl. Levels of ATP (12, 23, 31, 33), the extent of proton uptake (12, 18, 20, 33), and other phenomena indicative of ion movements (12, 18, 33) show no evidence for increases, only decreases in the presence of antimycin or NH_4Cl; thus, it is difficult to envision how a greater Mg^{2+} flux coupled to proton movement (13) or a greater alkalization of the stromal compartment can occur.

Alternatively, CO_2 fixation may be regulated by the rate of photosynthetic electron transport. Robinson and Wiskich (22) demonstrated that chloroplasts, like mitochondria, display a control of the electron transport process which is dependent on the supply of ADP or the presence of an uncoupler. Such a mechanism involving regulation of electron flow by the pH gradient (19) was suggested by Slovacek and Hind (30) for conditions where high light and lowered O_2 tension depressed the rate of HCO_3^--saturated photosynthesis; rate increases with either antimycin or uncouplers were prominent and greater as the ATP demand in the metabolism of the acceptor (*e.g.* HCO_3^-, PGA,[2] and OAA) was decreased. Accelerations by antimycin invariably occurred when rates of CO_2 fixation were repressed because of high levels of Pi (35), strong light intensities under anaerobic conditions (23, 24, 30), or when cat was omitted from aerobic samples (17, 18). It is conceivable that all of these inhibitory factors are related in that they decrease CO_2 fixation by disturbing the balance between ATP production and metabolic utilization in a manner which can be reversed by inhibiting photophosphorylation.

In this report, the effects of both NH_4Cl and antimycin are presented for a number of assay conditions shown to influence the rate of CO_2 fixation adversely.

MATERIALS AND METHODS

Intact chloroplasts were isolated from *Spinacia oleracea* as previously described (30). Measurement techniques for O_2 evolution or uptake and the flash-induced *A* changes due to Cyt b_6 (564 nm), Cyt *f* (554 nm), and P518 (518 nm) are detailed elsewhere

[1] This research was carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy.

[2] Abbreviations: PGA: 3-phosphoglycerate; OAA: oxaloacetate; cat: catalase; MV: methyl viologen; 9-AA: 9-aminoacridine; Rib-5-P: ribose 5-phosphate; Ru-5-P: ribulose 5-phosphate; RuBP: ribulose 1,5-bisphosphate; Fru-6-P: fructose 6-phosphate.

(30, 31). All assays were performed at 20 C with sample mixtures composed of chloroplasts in 3 ml reaction buffer containing 0.35 M sorbitol, 50 mM Tricine, and 0.25 mM K_2HPO_4, adjusted to pH 8.1 with NaOH or KOH. Experiments at pH 7.6 were performed with 50 mM Hepes in place of Tricine. Aerobic samples contained 1,600 units cat/ml unless specified. In O_2 uptake measurements with O_2 or MV, the cat was replaced by 0.6 mM KCN. The concentrations of electron acceptors routinely used were 10 mM $NaHCO_3$, 1.6 mM $NaNO_2$, and 30 μM MV. Chl, antimycin, and NH_4Cl concentrations are given in legends. For O_2 measurements, chloroplasts were normally illuminated with saturating intensities (1,200 w/m^2) of blue light (Corning 4-96). *A* changes were elicited by short (4-μs pulse width at half-peak height) red (Kodak Wratten 70) flashes provided at a frequency of 2.5 Hz by two EG&G FX201 xenon lamps. Simultaneous measurements of 9-AA fluorescence and O_2 evolution or uptake were performed in a single cuvette in the fluorimeter apparatus described by Mills *et al.* (18). Samples contained 10 μM 9-AA and were illuminated with 230 w/m^2 of red actinic light (Corning 2-58). The percentage of 9-AA uptake or fluorescence-quenching induced by actinic illumination was calculated as $Q = (Fl^D - Fl^L)/Fl^D$. Quenching was equivalent for both intact and osmotically ruptured chloroplasts illuminated at pH 8.1 with MV; hence, we can assume that fluorescence changes reflect predominantly the acridine dye distribution changes between the stroma and the thylakoid. Light-induced binding of the 9-AA probe to the thylakoid membrane was estimated to be about 22% in samples containing 22 μg Chl/ml and 5 μM monensin plus 25 mM Na^+ to abolish proton uptake completely. The binding term was similar to that previously measured by Tillberg *et al.* (33) of 1%/μg Chl and was subtracted before calculations of ΔpH. Using the equation of Schuldiner *et al.* (25), ΔpH was calculated as

$$\log \frac{Q}{1-Q} \cdot \frac{1}{V}$$

where V was taken as 3.3 μl/mg Chl times the mg Chl/ml of sample, based on the estimates of Heldt *et al.* (37) for the thylakoid-enclosed volume in intact chloroplasts. Antimycin A was purchased from Sigma.

RESULTS

Antimycin and NH_4Cl Effects on Electron Transport Activities. Figure 1 displays the effects of NH_4Cl concentration on HCO_3^--dependent O_2 evolution and MV-catalyzed O_2 uptake by intact chloroplasts. Linear electron flow to MV is substantially uncoupled at concentrations below 0.6 mM NH_4Cl in agreement with Heath and Leech (7). This is also consistent with the 30% decline in the ATP/2e ratio previously seen when ferricyanide reduction was uncoupled with 0.16 mM NH_4Cl in osmotically shocked chloroplasts (12). However, O_2 evolution with HCO_3^- as the acceptor is essentially unaffected in this range; only at concentrations above 0.6 mM NH_4Cl is the rate severely depressed. Evidently chloroplasts retain a capacity to balance the production rates of ATP and NADPH when linear electron flow is partially uncoupled. Presumably, increased phosphorylation associated with either pseudocyclic (8) or cyclic electron flow (1, 12, 31) occurs to offset the decreased coupling efficiency with $NADP^+$ as the acceptor and to maintain the stoichiometry required for reduction of CO_2.

In contrast to the results with NH_4Cl, Figure 2 shows that antimycin inhibits O_2 evolution with HCO_3^- over a range where both coupled and uncoupled electron flows to MV are unaffected. An antimycin-sensitive reaction, not involved in the linear electron transport sequence to MV, is clearly required for rapid rates of CO_2 fixation. Previous studies of intact chloroplasts have shown that antimycin decreases cyclic phosphorylation (12, 18, 31) and that the inhibition site apparently lies between Cyt b_6 and the

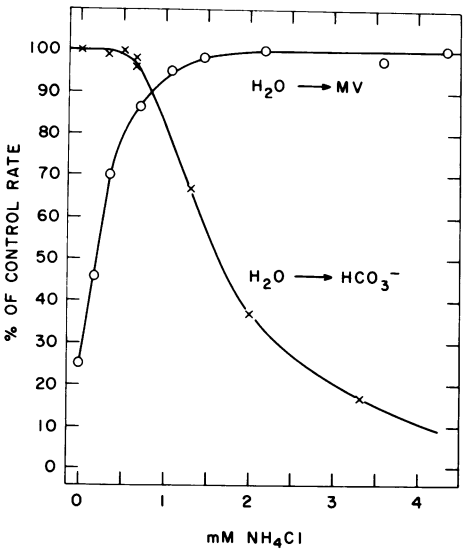

FIG. 1. Effects of NH_4Cl concentration on electron transport to HCO_3^- or MV. Chl concentration was 47 μg/ml; otherwise reaction conditions were as described under "Materials and Methods." Control rates were −251 and 122 μmol/mg Chl·h for O_2 uptake with MV or evolution with HCO_3^-, respectively.

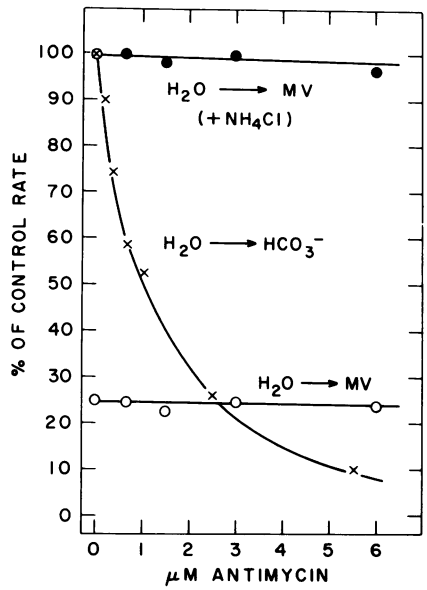

FIG. 2. Antimycin concentration effects on linear electron transport to HCO_3^- or MV. Conditions as in Figure 1 and "Materials and Methods." NH_4Cl concentration was 3.3 mM where indicated. Control rates were −208 for uncoupled O_2 uptake with MV and 122 μmol/mg Chl·h for O_2 evolution with HCO_3^-.

point at which cyclic electron flow enters the electron carrier sequence between PSI and PSII (31).

The responses of Cyt b_6 and Cyt *f* turnovers to NH_4Cl and antimycin are illustrated in Figure 3 for chloroplasts illuminated with repetitive flashes in the presence of HCO_3^-. The flash frequency (2.5 Hz) was insufficient to overcome the normal induction lag (35) and subsequently little net O_2 evolution was detected (data not shown). The reversible *A* changes due to Cyt b_6 and Cyt *f* must, therefore, reflect either cyclic or pseudocyclic electron flows. The ratio of 0.71 for Cyt b_6 to Cyt *f* heme turnover, calculated from the magnitude of the *A* changes under the control condition and the extinction coefficients of 20 and 22 $mM^{-1}cm^{-1}$,

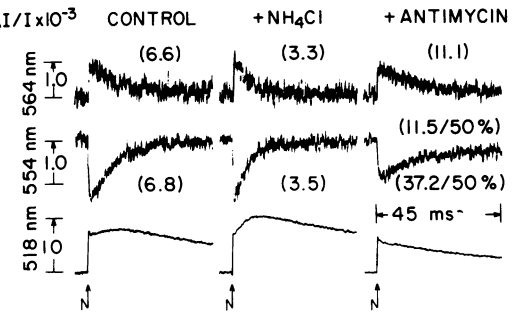

FIG. 3. Kinetic traces for flash-induced absorption changes attributable to Cyt b_6 (564 nm), Cyt f (554 nm), and the electrochromic effect (518 nm) during induction with HCO_3^-. Samples contained 50 μg Chl/ml, 10 mM $NaHCO_3$, and cat in reaction medium described under "Materials and Methods." Additions of antimycin or NH_4Cl were 1.0 μM and 0.33 mM, respectively. Numbers in brackets give the half-relaxation times in ms and when more then one relaxation time is involved, its percentage of the total signal is also given.

respectively (3), indicates that Cyt f activity is predominantly the result of cyclic rather than pseudocyclic electron flow. A low level of NH_4Cl (0.33 mM) promotes the more rapid oxidation of Cyt b_6 and reduction of Cyt f as seen from the decreases in the half-relaxation times for the A changes. The larger A transient, at 564 nm, increases the estimated Cyt b_6 to Cyt f turnover ratio to 0.93 and suggests that cyclic activity is more prominent in the presence of uncoupler. The slow rise in electrochromic effect (P518), which occurs milliseconds after the flash, is enhanced by the uncoupler and has recently been shown to reflect cyclic electron flow (4). Comparison with Figure 1 reveals that 0.33 mM NH_4Cl also increases the rate of coupled electron flow to MV by approximately 2-fold. In contrast, 1 μM antimycin decreases the reversible A changes and increases the half-relaxation times associated with Cyt b_6 oxidation and Cyt f reduction. It is noteworthy that 1 μM antimycin decreases the oxidation rate of Cyt b_6 by a factor of two, whereas O_2 evolution with HCO_3^- (Fig. 2) is also inhibited 2-fold. The results support the view that Cyt b_6 and Cyt f participate in coupled cyclic electron flow, but more importantly demonstrate the inhibitory action of antimycin and uncoupling effect of NH_4Cl on cyclic electron transfer under conditions which facilitate CO_2 fixation in intact chloroplasts.

Phosphorylation Contributions from O_2 and NO_2^- Reduction. Table I lists the rates of O_2 evolution and uptake when either NO_2^- or O_2 serves as the electron acceptor under conditions for coupled or uncoupled electron flow. It is apparent that near anaerobic NO_2^- reduction, mediated by electron flow through ferredoxin (11), is a tightly coupled process, as it responds to concentrations of NH_4Cl or antimycin that decrease the rate of ATP synthesis in intact chloroplasts (31). Similar results are seen when NO_2^- is added to aerobic samples containing cat. On the other hand, O_2 uptake, which is also thought to involve ferredoxin (8), is relatively unresponsive to uncoupler or antimycin additions. This reinforces the above conclusion that coupled pseudocyclic electron flow through Cyt f is minimal and that pseudocyclic ATP production is not a major factor in intact chloroplast carbon metabolism. Similar conclusions have been reached on the basis of simultaneous O_2 uptake and evolution studies with CO_2-fixing chloroplasts (8).

Enzymic reduction of NO_2^- does not require ATP, therefore, phosphorylation coupled to NO_2^- reduction can contribute to the ATP requirement of simultaneous CO_2 fixation. This property was used to investigate the ATP requirements during steady-state CO_2 fixation (Fig. 4). Comparison of traces a and e shows that the uncoupled rate of NO_2^- reduction is a significant fraction (approximately one-fifth) of the photosynthesis rate recorded when

Table I. *Electron Transport Rates with Nitrite or Oxygen as Electron Acceptors*

Additions to reaction medium containing chloroplasts (30 μg Chl/ml) were as indicated.

Acceptor	Conditions	Rate of O_2 Uptake or Evolution
		μmol/mg Chl·h
—	N_2 (<20 μM O_2)	0
1.6 mM $NaNO_2$	N_2 (<20 μM O_2)	14
1.6 mM $NaNO_2$	N_2 + 3.3 mM NH_4Cl	36
1.6 mM $NaNO_2$	N_2 + 1.0 μM antimycin	26
280 μM O_2	Air + cat	0
280 μM O_2 + 1.6 mM $NaNO_2$	Air + cat	13
280 μM O_2 + 1.6 mM $NaNO_2$	Air + 3.3 mM NH_4Cl	31
280 μM O_2	Air + 0.6 mM KCN	−14
280 μM O_2	Air + 3.3 mM NH_4Cl	−16
280 μM O_2	Air + 1.0 μM antimycin	−15

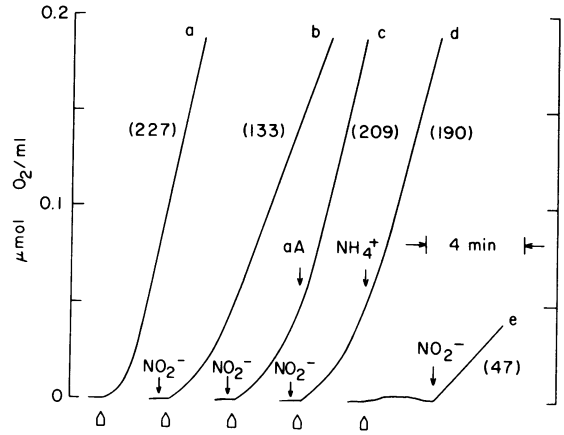

FIG. 4. Nitrite effects on O_2 evolution with HCO_3^-. Traces a, b, c, and d initially contained 10 mM $NaHCO_3$, although traces b, c, and d also contained 1.6 mM $NaNO_2$. Arrows indicate addition of either 0.16 μM antimycin A, 0.33 mM NH_4Cl (NH_4^+), or 1.6 mM $NaNO_2$ (NO_2^-). Trace e had 3.3 mM NH_4Cl substituted for $NaHCO_3$. Chl concentration was 17 μg/ml. Numbers in brackets give the steady-state rates of O_2 evolution in μmol O_2/mg Chl·h; arrows at bottom of traces indicate the onset of illumination.

only HCO_3^- is present. Nitrite reduction could contribute significantly to ATP production given an ATP/2e ratio of 1.33 for linear electron flow (12, 19). A substantial drop (42%) in the rate of O_2 evolution occurs when both HCO_3^- and NO_2^- are present from the onset as seen in trace b and documented elsewhere by others (6, 11, 20). Additions of antimycin (trace c) or NH_4Cl (trace d) nearly restore the inhibited rate to that observed with HCO_3^- alone. It is possible that NH_4Cl may relieve the NO_2^- inhibition by causing a greater alkalization of the stroma, but it is clear from Figures 1 and 3 that such low levels of NH_4Cl also uncouple electron flow. Antimycin inhibits proton uptake without uncoupling (18), hence it is more likely that similar accelerations by antimycin and NH_4Cl are related to their common effects of inhibiting ATP production by decreasing the pH gradient.

Effects of NH_4Cl and Antimycin under Suboptimal Assay Conditions. Independent studies have shown that CO_2 reduction is maximal if low substrate concentrations of Pi are used (14, 35), cat is added to prevent H_2O_2 buildup (15) and the reaction medium pH is fixed near 8.1 (7, 14). The reaction conditions described

under "Materials and Methods" and used in the previous experiments were designed to accomodate these factors. In the following studies, assay conditions were systematically varied and the effects of partially uncoupling NH_4Cl concentrations or partially inhibitory levels of antimycin were compared to those observed in the optimal control case.

Figure 5 depicts examples of O_2 evolution traces recorded under optimal and selected suboptimal conditions for CO_2 fixation. In the control, 0.33 mM NH_4Cl does not affect O_2 evolution, whereas 0.33 μM antimycin decreases the rate by approximately 25%. If cat is omitted from samples, the rate is decreased and can be restored by additions of either NH_4Cl or antimycin. Chloroplasts incubated in the presence of cat and 2.0 mM K_2HPO_4 display a longer induction period and a lower steady-state rate, as previously shown by Walker (35). The low rate is accelerated by the inclusion of either antimycin or NH_4Cl. These findings confirm some earlier observations by Walker (14) and Mills *et al.* (18) and further demonstrate that antimycin and NH_4Cl are equally effective in restoring high rates of O_2 evolution in chloroplasts subjected to high phosphate inhibition or H_2O_2 poisoning.

The concentration optimum for acceleration of O_2 evolution by antimycin in chloroplasts without added cat is presented in Figure 6. A comparison with the control indicates that about 25% inhibition of cyclic activity is necessary to restore the rate in samples without added cat. Both the acceleration effect and the decreased sensitivity at higher antimycin concentrations imply that cyclic ATP production is excessive under conditions where H_2O_2 buildup is allowed to occur. Similar findings have been reported for the effects of antimycin under anaerobic conditions (12).

Table II summarizes the O_2 evolution capabilities of chloroplasts subjected to a range of assay conditions with saturating levels of HCO_3^- as the electron acceptor. In the first column are listed the reaction parameters and in the second column, the normalized rates of O_2 evolution. In agreement with earlier reports (references in column 3), antimycin and uncouplers accelerate rates of O_2 evolution, but only under conditions which normally repress the rate of photosynthesis. The major inhibitory factors are cat omission, high levels of K_2HPO_4, inclusion of NO_2^-, or lowered O_2 tension, and may be used alone or in combinations. Incubation of chloroplasts at pH 7.6, rather than 8.1, under otherwise optimal conditions, is not sufficient to alter markedly the rate of O_2 evolution, and the effects of low NH_4Cl or antimycin concentrations are marginal. High rates of O_2 evolution have routinely been documented at pH 7.6 (8, 14, 15, 20, 35), so it is more likely that cat omission (15) or excessive phosphate at this pH (14, 35) or a combination of both is responsible for the low rates reported occasionally (7, 17).

The pattern, evident in Table II, is one in which inhibitors or uncouplers of cyclic photophosphorylation are required to restore high rates of O_2 evolution in chloroplasts assayed under less than optimal conditions. Further evidence for a consistent pattern is provided in other observations (not reported in the table) that mM levels of Rib-5-P also bring about an accelerated rate of CO_2 fixation under each of the four suboptimal conditions defined here (6, 14, 15, 21, 30, 36). Slabas and Walker (29) have shown

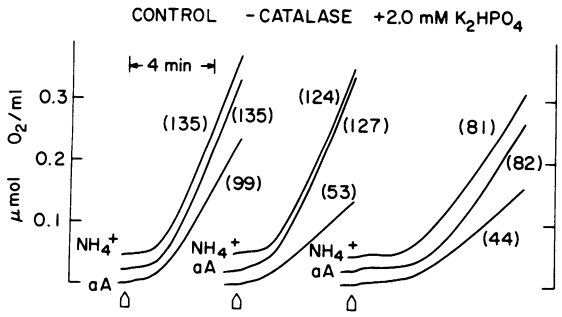

FIG. 5. O_2 evolution traces under optimal and suboptimal assay conditions for HCO_3^- reduction. Concentrations of Chl, antimycin (aA), and NH_4Cl (NH_4^+) were 40 μg/ml, 0.33 μM, and 0.33 mM, respectively.

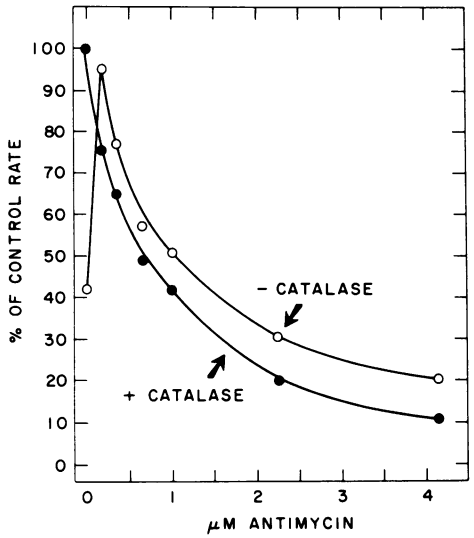

FIG. 6. Antimycin concentration curves for HCO_3^--dependent O_2 evolution in samples illuminated with and without cat. Chl concentration was 32 μg/ml; otherwise conditions were as in Figure 2 and "Materials and Methods." Maximum control rate was 152 μmol O_2/mg Chl·h.

Table II. *Summary of HCO_3^--Dependent O_2 Evolution Rates in Response to NH_4Cl or Antimycin Additions under Different Assay Conditions*

LP_i, HP_i, and cat indicate the presence of 0.25 mM or 2.0 mM K_2HPO_4 and cat (1,600 units/ml), respectively. $NaNO_2$ (NO_2^-) concentration was 1.6 mM. NH_4Cl and antimycin A (aA) concentrations as indicated. Relative rates were standardized against control rates (120–220 μmol O_2/mg Chl·h) observed under optimal conditions. LP_i: low inorganic phosphate; HP_i: high inorganic phosphate.

	Conditions	Relative Rate	References
Optimal	Air, pH 8.1, LP_i, +cat	100	18, 30
	Air, +NH_4Cl 0–0.5 mM	100	
	Air, +aA 0.1–1.0 μM	90–50	18, 30
Near optimal	Air, pH 7.6, LP_i, +cat	85	15, 20, 36
	Air, +NH_4Cl 0.5 mM	100	
	Air +aA 0.16 μM	90	36
Suboptimal	Air, pH 8.1, LP_i, −cat	40	18
	Air, +NH_4Cl 0.33 mM	92	
	Air, +aA 0.33 μM	94	18
	Air, pH 7.6, LP_i, −cat	30	7, 14–16
	Air, +NH_4Cl 0.33 mM	90	7, 14
	Air, +aA 0.16 μM	85	17
	Air, pH 8.1, HP_i, +cat	33	18, 36
	Air, +NH_4Cl 0.33 mM	60	
	Air, +aA 0.33 μM	61	18, 36
	Air, pH 8.1, LP_i, +cat +NO_2^-	58	20
	Air, +NH_4Cl 0.5 mM	84	20
	Air, +aA 0.16 μM	92	
	N_2, pH 8.1, LP_i ± cat	37	6, 23, 24, 30
	N_2, +NH_4Cl 0.33 mM	78	30
	N_2, +CCCP 0.16 μM	74	30
	N_2, +aA 1.0 μM	81	23, 24, 30
	N_2, pH 8.1, LP_i −cat +NO_2^-	27	6
	N_2, +aA 1.0 μM	58	

that substrate additions of Rib-5-P may act as an ATP sink by conversion to Ru-5-P and RuBP.

Regulation of Electron Transport by a pH Gradient. To determine whether excessive phosphorylation and the subsequent buildup of a proton gradient back pressure might restrict electron flow under the inhibitory conditions noted in Table II, the effective range for ΔpH regulation of electron transport was determined from experiments described in Figure 7. Traces a and b show that O_2 uptake is accelerated 3.3-fold while quenching of 9-AA fluorescence is reduced from 90 to 65%. In trace c, the results obtained under optimal CO_2-fixing conditions are presented for comparison and illustrate that attainment of steady-state O_2 evolution is accompanied by a small decline in the level of 9-AA quenching to approximately 61%. Similar measurements (33) with chloroplasts incubated at a pH below the light driven stromal pH gave smaller changes and were correlated with a ΔpH between the thylakoid and external medium. To estimate more accurately ΔpH between the intrathylakoid and stromal spaces, experiments here were performed at pH 8.1 as it was shown (37) that light-induced alkalization of the stroma is not significantly different from the medium at this pH.

The relationship between quenching of 9-AA fluorescence or ΔpH and the rate of O_2 uptake with MV is plotted in Figure 7d and was obtained by varying the level of either NH_4Cl or monensin. The largest changes in electron transport are correlated with relatively small changes in quenching and occur at quenching values exceeding 65% or a ΔpH of 4.0 units.

In Table III, the percentage of fluorescence-quenching and estimated ΔpH is listed for selected CO_2-fixing conditions. From Figure 7d and Table III, it is evident that the pH gradient is sufficient to drive phosphorylation during CO_2 fixation, but normally lies within a transition region between minimal and maximal ΔpH values for control of the electron transport process. Omission of cat or additions of either NO_2^- or excess K_2HPO_4, increase ΔpH into a region where electron transport becomes critically dependent upon small changes (0.1–0.2 unit) in ΔpH. Further additions of antimycin or NH_4Cl restore ΔpH to a value similar to that observed under conditions for maximal CO_2 fixation rates. Note that absolute rate changes documented in Figures 4 and 5 as well as Table II are within the range of rates observed in Figure 7 where ΔpH was systematically varied between 3.5 and 4.5 pH units. Both the inhibitions of HCO_3^--saturated photosynthesis under varied assay conditions and the restored rates with NH_4Cl or antimycin can be ascribed directly to a pH-dependent control of the electron transport process.

Additional evidence for this explanation is presented in Table IV where CO_2-dependent O_2 evolution was measured at decreasing light intensities and hence, lower transthylakoid pH gradients. For samples with cat present it can be seen that as the illumination is lowered and NH_4Cl added to further reduce ΔpH, a larger rate decrease is observed relative to the control case. In contrast, the inhibition produced by cat omission becomes less severe and rate accelerations with either NH_4Cl or antimycin are also diminished. Strong illumination is evidently required to maximize both the rate inhibition without added cat and the rate increases seen with

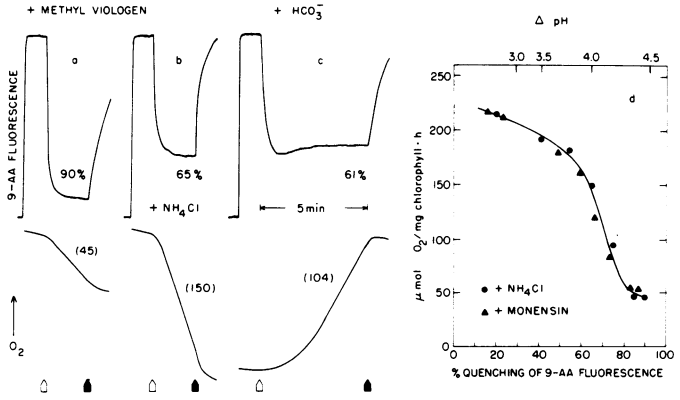

FIG. 7. Electron transport rates *versus* ΔpH in intact chloroplasts. All samples contained 10 μM 9-AA and 22 μg Chl/ml in reaction buffer (pH 8.1). Trace a contained 25 μM MV and 1.0 mM KCN, although in trace b, 0.5 mM NH_4Cl was added. Trace c contained 10 mM $NaHCO_3$ and cat. In part d, the rate of O_2 uptake and 9-AA fluorescence-quenching was varied by increasing the levels of either NH_4Cl (●) or monensin (▲) added in the ranges 0–2.5 mM and 0–5.0 μM, respectively, to samples containing MV. ΔpH was calculated after correction for the binding term as described under "Materials and Methods."

Table III. *Summary of ΔpH Determinations under Different Assay Conditions for CO_2 Fixation*

Measurements of 9-AA fluorescence-quenching (% Q) and estimated ΔpH as described in "Materials and Methods." All samples contained 10 μM 9-AA, 10 mM HCO_3^-, and 22 μg Chl/ml. Additional abbreviations as indicated in Table II. Control rate of O_2 evolution with 10 mM HCO_3^- and cat at pH 8.1 was 104 μmol O_2/mg Chl·h.

Conditions	Observed	pH
	% Q	Δ
Air, pH 8.1, LP_i, +cat	61–66	3.94–4.00
Air, pH 8.1, +aA 0.25 μM	58	3.89
Air, pH 8.1, +NH_4Cl 0.25 mM	54	3.81
Air, pH 8.1, +NH_4Cl 0.50 mM	47	3.66
Air, pH 8.1, LP_i, −cat	78	4.24
Air, pH 8.1, +aA 0.25 μM	66	4.00
Air, pH 8.1, +NH_4Cl 0.25 mM	63	3.98
Air, pH 8.1, LP_i, +cat +NO_2^-	75	4.19
Air, pH 8.1, +aA 0.25 μM	68	4.06
Air, pH 8.1, +NH_4Cl 0.25 mM	61	3.94
Air, pH 8.1, HP_i, +cat	70	4.10
Air, pH 8.1, +aA 0.33 μM	62	3.96
Air, pH 8.1, +NH_4Cl 0.25 mM	52	3.77

Table IV. *Light Intensity Dependence for the Effects of NH_4Cl and Antimycin*

Samples contained 10 mM HCO_3^- and 23 μg Chl/ml in reaction medium. Blue illumination was provided at intensities listed. Additions of cat, NH_4Cl, and antimycin (aA) were 1,600 units/ml, 0.33 mM, and 0.33 μM, respectively.

Light Intensity	Conditions	Rate of O_2 Evolution	Control
w/m^2		μmol/mg Chl·h	%
1,000	+cat	80	100
	+cat + NH_4Cl	74	93
	−cat	28	35
	−cat + NH_4Cl	55	69
	−cat + aA	52	65
236	+cat	57	100
	+cat + NH_4Cl	52	91
	−cat	23	40
	−cat + NH_4Cl	36	63
	−cat + aA	33	58
117	+cat	38	100
	+cat + NH_4Cl	25	66
	−cat	21	55
	−cat + NH_4Cl	25	66
	−cat + aA	22	58
49	+cat	20	100
	+cat + NH_4Cl	10	50
	−cat	11	55
	−cat + NH_4Cl	11	55
	−cat + aA	11	55

antimycin or NH_4Cl. These effects are similar to those reported for anaerobic chloroplasts (30) and apparently do not occur at low light intensities where a lower rate of proton-pumping and subsequently ATP formation limits the over-all process of CO_2 fixation.

Studies at Ambient CO_2 Levels. Another factor which lowers the over-all rate of CO_2 assimilation is the removal of the Calvin cycle intermediate RuBP by oxidative glycolate formation at ambient levels of dissolved CO_2 (*i.e.* 11 μM at 20 C) under aerobic conditions (28). To approximate this *in vivo* situation, chloroplasts were supplemented with 0.63 mM $NaHCO_3$ (11 μM CO_2 at pH 8.1 and 20 C) plus exogenous cat. O_2 evolution was recorded as shown in Figure 8. With ambient CO_2, in trace b, the maximum rate of O_2 evolution is only 30% of the rate observed with 10 mM $NaHCO_3$ (180 μM CO_2 at pH 8.1) in trace a. Addition of 5 mM $NaHCO_3$ (90 μM CO_2) restores the rate almost to that with saturating HCO_3^- present initially. If 1.0 mM Rib-5-P is included, the inhibition is partially reversed (trace c) and the results are qualitatively similar to those of Robinson and Gibbs (21) at low CO_2 concentrations; further addition of 5 mM $NaHCO_3$ completely restores the maximum rate of photosynthesis. If 0.33 mM NH_4Cl is added, as in trace d, the rate is lowered by 50%, in contrast to the lack of effect seen at high HCO_3^- (cf. Figs. 1 and 5) or when additional HCO_3^- is added after presentation of the uncoupler. Similarly, antimycin greatly decreases the CO_2-limited rate, though this inhibition is not fully reversed by 5 mM $NaHCO_3$ (in agreement with Figs. 2 and 5). Supplementation with NO_2^- accelerates O_2 evolution (Fig. 8, trace f) but at a saturating HCO_3^- level, NO_2^- appears as an inhibitor as seen earlier (Fig. 4). The results of Figure 8 show that ATP production under ambient CO_2 may be insufficient to support a maximal rate of photosynthesis, in contrast to Tables II and III, where ΔpH and phosphorylation appeared adequate or excessive. At low CO_2 levels, studies with 9-AA gave no indication for an increased ΔpH over that observed for the control case with high CO_2.

Conclusions

Previous proposals for indirect modulation of enzyme activities and subsequent regulation of CO_2 fixation (7, 17, 20, 23, 24, 36) are based on the assumption that coupled electron transport phenomena are not rate-limiting and are virtually unaffected by low concentrations of NH_4Cl or antimycin. The assumption is questionable in view of these electron transport studies which demonstrate uncoupling of both linear and cyclic electron flows with less than 0.5 mM NH_4Cl and inhibition of cyclic electron transfer between Cyt b_6 and Cyt f by antimycin. Both compounds have been shown to decrease ΔpH across the thylakoid membrane and lower the phosphorylation activity of chloroplasts (12) at the levels employed here. Any explanation for the effects of antimycin or NH_4Cl on CO_2 fixation rates must be consistent with their common inhibitory effect (albeit by different means) on proton gradient formation and ATP production.

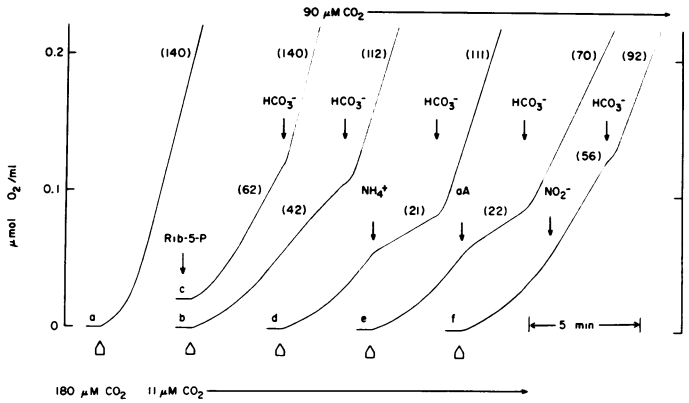

FIG. 8. O_2 evolution traces at ambient and high levels of CO_2. Trace a contained 10 mM $NaHCO_3$ (180 μM CO_2 at pH 8.1 and 20 C) from the onset of illumination (open arrow). Traces b and c contained 0.63 mM $NaHCO_3$ (11 μM CO_2). Additions of Rib-5-P (1 mM), HCO_3^- (5 mM), NH_4^+ (0.33 mM), antimycin *A* (0.33 μM), and NO_2^- (1.5 mM) are indicated by solid arrows. Numbers in parentheses give rates of O_2 evolution in μmol/mg Chl·h.

In each of the suboptimal cases examined when saturating $NaHCO_3$ was present, the requirement for inhibitors of phosphorylation to restore high rates of O_2 evolution strongly suggests a major imbalance between the rates of ATP production and utilization. Large increases in the control ATP/ADP ratios, after NO_2^- addition or in the case of H_2O_2 poisoning, have been observed by Heldt *et al.* (10). Phosphorylation coupled to NO_2^- reduction (11) or increased cyclic activity under high light and anaerobiosis (30) could plausibly produce such an imbalance by increasing ATP synthesis without subsequent changes in NADPH production and its metabolic consumption rate. Alternatively the perturbation could arise from a decrease in the metabolic demand for ATP. Heldt *et al.* (9) and Steup *et al.* (32) have recently shown that both CO_2 reduction and starch accumulation by chloroplasts is decreased with increasing levels of Pi. A decline in starch synthesis would lower the net ATP requirement for CO_2 assimilation (34) independent of the mechanism proposed to control starch synthesis.

Inhibition of CO_2 fixation by H_2O_2 poisoning has been located in the transketolase reaction involving Fru-6-P (16, 27). Direct inhibition of enzymic activity (15) could lead to a decline in the pentose-P precursors to Ru-5-P and the ATP consumption reaction which produces RuBP. Alternatively, H_2O_2 oxidation of the transketolase-glycolaldehyde complex to produce glycolate (27) would also inhibit regeneration of pentose-P and possibly decrease the pool of Fru-6-P available as a starch precursor. Both mechanisms would serve to lower the demand for ATP.

Accelerations in the rate of CO_2 reduction, under each of the above conditions, would require a partial decrease in ΔpH and, consequently, the rate of phosphorylation to restore the balance in ATP production and consumption. This view is consistent with the principle effects of both NH_4Cl and antimycin on ΔpH, photophosphorylation, changes in CO_2 fixation rates, and with metabolic labeling patterns (5, 21, 23, 24, 26).

When less than saturating levels of CO_2 are present, the oxygenase function of RuBP carboxylase converts RuBP into glycolate-P and PGA (28). At ambient levels of CO_2, this removal of carbon from the Calvin cycle might be considered analogous to the case where H_2O_2 oxidation of the transketolase-glycolaldehyde complex leads to glycolate or when triose phosphates are exported under high phosphate. However, there is a major difference; the carbon removed at low CO_2 is derived from RuBP after all of the phosphorylation reactions have occurred, whereas triose-P or Fru-6-P removal occurs before the final phosphorylation step. Accordingly, CO_2 fixation at physiological CO_2 and O_2 levels would present a greater demand for ATP and should be more sensitive to the inhibition of phosphorylation as is confirmed by the observations presented here.

This study indicates that energetic interplay, between the electron transport reactions responsible for ATP and NADPH production and the enzymic processes which utilize these cofactors during carbon metabolism, is an important regulatory feature of chloroplast photosynthesis. More detailed studies of carbon flow through the Calvin cycle are needed to clarify such interactions.

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