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Influence of Antimycin A and Uncouplers on Anaerobic Photosynthesis in Isolated Chloroplasts[1]

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ABSTRACT

Anaerobiosis depresses the light- and bicarbonate-saturated rates of O_2 evolution in intact spinach (*Spinacia oleracea*) chloroplasts by as much as 3-fold from those observed under aerobic conditions. These lower rates are accelerated 2-fold or more by the addition of 1 μM antimycin A or by low concentrations of the uncouplers 0.3 mM NH_4Cl or 0.25 μM carbonyl cyanide *m*-chlorophenylhydrazone. Oxaloacetate and glycerate 3-phosphate reduction rates are also increased by antimycin A or an uncoupler under anaerobic conditions. At intermediate light intensities, the rate accelerations by either antimycin A or uncoupler are inversely proportional to the adenosine 5'-triphosphate demand of the reduction process for the acceptors HCO_3^-, glycerate 3-phosphate, and oxaloacetate. The acceleration of bicarbonate-supported O_2 evolution may also be produced by adding an adenosine 5'-triphosphate sink (ribose 5-phosphate) to anaerobic chloroplasts. The above results suggest that a proton gradient back pressure resulting from antimycin A-sensitive cyclic electron flow is responsible for the depression of light-saturated photosynthesis under anaerobiosis.

Photosynthetic CO_2 fixation requires that intact chloroplast electron transport supply 3 moles of ATP and 2 moles of NADPH for each mole of CO_2 fixed in the Calvin cycle (4). However, uncertainty in the *in vivo* coupling ratio (11, 26) and the fact that average quantum requirements of 10 to 12 quanta/CO_2 fixed (10) exceed the expected value of 8, provide doubt that 3 ATP molecules may be formed for every 2 NADP reduced by the linear scheme of photosynthetic electron transport (26). If insufficient ATP is generated during NADP reduction, the extra quanta must be required in some other coupled photoreaction. There is evidence (9) that O_2 reduction may be coupled to ATP formation through a portion of the electron carrier sequence normally involved in NADP reduction. A ferredoxin-dependent cycle around PSI has been shown to generate ATP under anaerobic conditions (3) and may also function aerobically *in vivo*. Several investigators (3, 14, 25, 26) have suggested the existence of a cycle in which electrons from the primary acceptor of PSI reduce Cyt *b*-563 and are subsequently returned to the oxidized PSI trap, $P700^+$, in a process coupled to the production of ATP. The inhibitor antimycin A of mitochondrial *b* and *c* type Cyt interaction (23) has previously been used to study the function of *b* type Cyt in both bacterial and green plant cyclic electron transport (1, 3, 13-15, 19).

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In broken chloroplasts, antimycin A is a reported inhibitor of linear electron transport (8) and a potent inhibitor of ferredoxin-catalyzed cyclic photophosphorylation (3). In addition, the antibiotic may function as an uncoupler when used at higher concentrations (8, 13). However, in intact chloroplasts, antimycin A increases the rate of CO_2 reduction under anaerobic conditions (21, 22). Aerobically, antimycin A also accelerates CO_2 fixation if suboptimal conditions (*i.e.* high Pi concentrations) are imposed upon the system (27). This effect is hard to reconcile with either an inhibition of NADP reduction or a decreased rate of phosphorylation, and present hypotheses concerning the antimycin A acceleration of CO_2 fixation have in common an indirect activation of carbon metabolism. Schacter *et al.* (22) first suggested that antimycin A affected an enzyme mechanism which controlled the rate of CO_2 fixation. Schacter and Bassham (21) later proposed that ribulose-1,5-diP carboxylase and fructose 1,6-diphosphatase activity might be indirectly enhanced by antimycin A. Miginiac-Maslow and Champigny (18) recognized that a decreased phosphorylation potential could accelerate NADP reduction but further suggested that increased enzyme activity might be the result of stromal pH and Mg^{2+} ion levels responding to accelerated electron transport. On the basis of enzyme activation studies, Champigny *et al.* (7) concluded that light activation of P-ribulokinase was enhanceable by antimycin A. More recently, Walker *et al.* (28) proposed that fructose 1,6-diphosphatase activity was enhanced by antimycin A through a direct increase in the level of reduced ferredoxin. This suggestion was based on the findings of Buchanan *et al.* (6) that light activation of fructose 1,6-diphosphatase was mediated through reduced ferredoxin.

This investigation is an attempt to determine whether increased CO_2 reduction rates might be causally related to the inhibitory action of antimycin A on cyclic electron transport.

MATERIALS AND METHODS

Material. Spinach (*Spinacia oleracea*, Hybrid 424, Park Seed Co.) was grown in chambers with a 10 hr, 27 C day and a 14 hr, 24 C night period. An incident intensity of 60 w m^{-2} was obtained with a combination of 5,160 w fluorescent and 3,000 w incandescent light. Leaf material from 4- to 8-week-old plants was harvested 1 to 2 hr into the photoperiod.

Chloroplast Isolation. The isolation procedure was similar to that of McC. Lilley and Walker (17) except for the following modifications. Approximately 60 g of leaves were chilled in ice water, deveined and shredded into 125 ml of semifrozen buffer containing 0.36 M sorbitol, 15 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, and 3 mM sodium isoascorbate at pH 6.5. The preparation was ground for 1 sec at full power and for 2 sec at half-power in a Waring Mini-Blendor equipped with a double-edged razor blade. The mash was filtered through eight layers of cheesecloth premoistened with the cold isolation buffer. The filtrate was

centrifuged from zero to 4,000*g* and back to zero in one continuous accelerating motion which lasted approximately 90 sec. The pellet was washed with and resuspended in buffer containing 0.36 M sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ at pH 7.55. Chl content was determined by the method of Arnon (2).

Photosynthetic Assays. Measurements of O_2 evolution were performed polarographically with a thermally jacketed (18 C) Clark electrode cell (Yellow Springs Instrument Model No. 5331). Sample mixtures were composed of 40 to 45 μg Chl·ml^{-1} in 3 ml of reaction buffer containing 0.36 M sorbitol, 50 mM Tricine, and 0.5 mM K_2HPO_4 adjusted to pH 8.15 with KOH. The concentrations of the electron acceptors were typically 15 mM $NaHCO_3$, and 5 to 7 mM for either PGA[2] or OAA. Aerobic samples routinely contained 1,600 units of catalase/ml to prevent the buildup of H_2O_2 (16). Whereas aerobic rates were optimal in the presence of added catalase, the presence or absence of catalase had no effect on the anaerobic samples. Near-anaerobic samples (less than $^1/_{10}$ the concentration of O_2 in air-saturated water) were produced by flushing the reaction buffer with N_2 prior to the addition of chloroplasts. Illumination was provided by a 500 w slide projector and the appropriate heat filter and condensing lens system. Light intensities are given in the figure and table legends. Rates reported in the figures and tables represent steady-state values recorded in the 3rd to 4th min of illumination (see Fig. 5 for examples of the induction kinetics for O_2 evolution). The results presented here are from chloroplast preparations which were greater than 70% intact as assayed by the ferricyanide method (12).

RESULTS

Figure 1 displays the effect of anaerobicity and light intensity on the acceleration by antimycin A of bicarbonate-supported O_2 evolution. In the control curves, the aerobic and anaerobic rates are about equal at low light intensity but diverge steadily as the intensity rises and have different saturation points so that, at the highest intensity studied, the anaerobic rate is only a third of the aerobic rate. This absolute aerobic rate is within range of the highest so far reported for isolated chloroplasts (11, 17) whereas the anaerobic rate closely approximates the control values reported in prior studies of anaerobic CO_2 fixation (21, 22). These curves indicate that a photoreaction requiring anaerobicity and high light intensity depresses the rate of O_2 evolution. Data presented later will show that this depressed rate is nonetheless linear and cannot be ascribed to irreversible photoinactivation. Addition of antimycin A at low light intensities inhibits the rate of O_2 evolution, but at higher intensities it alleviates inhibition of the anaerobic rates and the curves run below and parallel to the aerobic control. An experimental feature which should be noted is that biphasic intensity curves such as those in Figure 1, clearly suggesting the involvement of a high intensity photoreaction, are obtained only with absolute intensities higher than the commonly accepted saturation values of 250 to 800 w m^{-2} (10, 12, 17).

To determine whether the release of inhibition by adding antimycin A is specific for bicarbonate reduction, or is a general property of anaerobic electron transport, intensity curves were run using PGA and OAA as acceptors. Figure 2 shows that with PGA as the terminal acceptor, O_2 evolution is also accelerated by antimycin A at high light intensities. The reduction of PGA requires only two Calvin cycle enzymes, 3-P-glycerate kinase and glyceraldehyde-3-P dehydrogenase plus a supply of ATP and NADPH. Thus, a major effect of antimycin A must be on one or more of these components. A direct photoactivation of 3-P-glycerate kinase may be ruled out by previous work

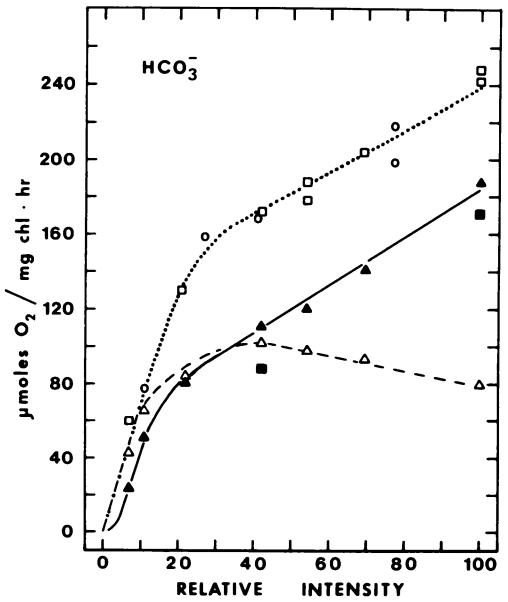

FIG. 1. Intensity curves for aerobic and anaerobic O_2 evolution with HCO_3^-. Dotted line through open symbols indicates the rates observed under aerobic (≧280 μM O_2) conditions with either orange (○) or white (□) light. Rates observed under initially anaerobic (<20 μM O_2) conditions in white light are given by dashed line through open triangles (△). Solid line through closed triangles (▲) indicates anaerobic rates in the presence of 1 μM antimycin A. Solid squares (■) give aerobic rates with 1 μM antimycin A. Absolute intensity at 100% was equal to 5000 w m^{-2}. Chl concentration was 43 μg Chl·ml^{-1} and HCO_3^- 15 mM.

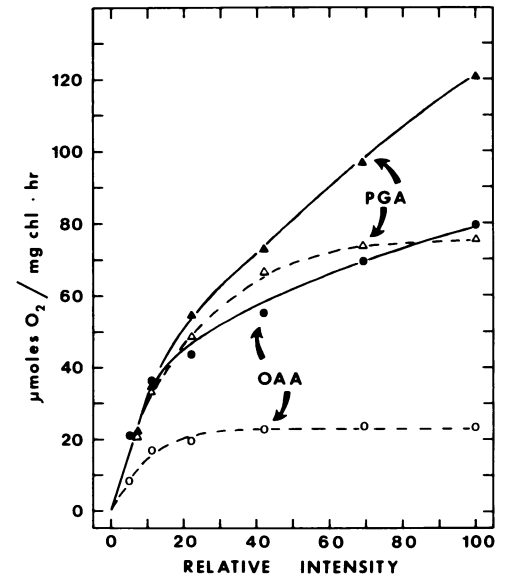

FIG. 2. Intensity curves for anaerobic O_2 evolution with 3-P-glycerate and OAA. Rates with 5 mM PGA are given by triangles (△, ▲) and those with 5 mM OAA by circles (○, ●). Dashed lines through open symbols (△, ○) represent controls while solid lines through closed symbols (▲, ●) represent rates in the presence of 1 μM antimycin A. Other reaction conditions were as described in Figure 1.

(7) which has shown that this enzyme is regulated by the energy charge in the adenylate pools. Alternatively, the 3-P-glycerate kinase reaction may be controlled by simple equilibrium effects (27, 28). Viewed either way, the conversion of PGA to glycerate-1,3-diP (DPGA) could only be accelerated if the over-all ATP level was increased by antimycin A, not decreased as experimentally is the case (3, 18, 21). Therefore, the acceleration of PGA reduction by antimycin A may be limited to an

[2] Abbreviations: PGA: glycerate 3-phosphate; OAA: oxaloacetate; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone.

effect on either the glyceraldehyde-3-P dehydrogenase or the supply of NADPH.

Antimycin A accelerates the low anaerobic rates of OAA reduction (Fig. 2) as much as 4-fold, to values approaching those for PGA reduction. This remarkable result removes from consideration any proposed interaction, direct or otherwise, between antimycin A and enzymes of the carbon pathway in interpreting the stimulatory effects of this antibiotic, since OAA reduction involves only NADPH and an enzyme which does not function in the Calvin cycle (malate dehydrogenase). The data from Figures 1 and 2 are instead consistent with the proposal that anaerobicity depresses NADP reduction, especially at high light intensity, and that antimycin A somehow relieves this inhibition. Furthermore, this antimycin A effect is more pronounced as the ATP demand of the terminal acceptor system decreases.

At low light intensities (Fig. 2) O_2 evolution rates with PGA as the electron acceptor are not inhibited by antimycin A which, in view of the ATP requirement for PGA reduction, suggests that antimycin A is not drastically lowering the ATP supply as would be the case if uncoupling occurred at the concentration employed.

The concentration dependences of antimycin A effects on anaerobic PGA and OAA reduction have not previously been documented. Figure 3 displays this function for all three acceptor systems under equivalent experimental conditions. The reduction of bicarbonate has the largest demand for ATP relative to NADPH (3/2) and has a sharp optimum at about 1 μM, as noted earlier (22). Both PGA- and OAA-dependent O_2 evolutions are similarly enhanced by 1 to 2 μM antimycin A but PGA reduction is less inhibited and OAA reduction is slightly stimulated by the highest concentrations studied. This is consistent with the known uncoupling activity of antimycin A at high concentrations (8, 13) and the small or zero dependences on ATP supply of PGA or OAA reduction, respectively.

One factor known to control NADP reduction is the degree of coupling between electron transport and formation of a transmembrane pH gradient (20). Previous studies have shown that OAA reduction, because it does not utilize ATP, is inhibited if the consequent back pressure of the pH gradient on electron transport is not relieved by adding an uncoupler (11). The acceleration of OAA reduction by antimycin A is phenomenologically similar to the action of an uncoupler, although antimycin A does not appear to inhibit or uncouple noncyclic electron flow at such a low concentration (8). Its most probable site of action is within the cyclic electron transport pathway. On the basis of early data (3) and the reputed involvement of autooxidizable carriers (5, 24), anaerobic conditions would be expected to favor cyclic electron transport and thereby help sustain a rate-limiting pH gradient.

If both antimycin A and uncouplers promote anaerobic OAA reduction by partially lowering the pH gradient it is reasonable to propose that uncouplers might accelerate anaerobic PGA and CO_2 reductions, as does antimycin A. Because of the need for some ATP these latter acceptor systems would be expected to show sharp optima in the uncoupler concentration curves, above which the ATP supply would be rate-limiting. These expectations are fulfilled, as shown for CO_2 reduction in Figure 4. The inset demonstrates that uncoupler does not merely provoke a short burst of O_2 equivalent to the NADP pool, but gives sustained higher rates indicative of enhanced CO_2 fixation. Since all of the experiments reported here are performed in suspensions buffered to pH 8.15, it is improbable that the accelerated rates result from changes in stroma pH, which itself is optimal at this value (29). The fact that ammonium chloride, CCCP, and antimycin A produce nearly identical effects suggests that the increased rates are not a function of a specific uncoupling mechanism as was previously proposed (29) to explain the

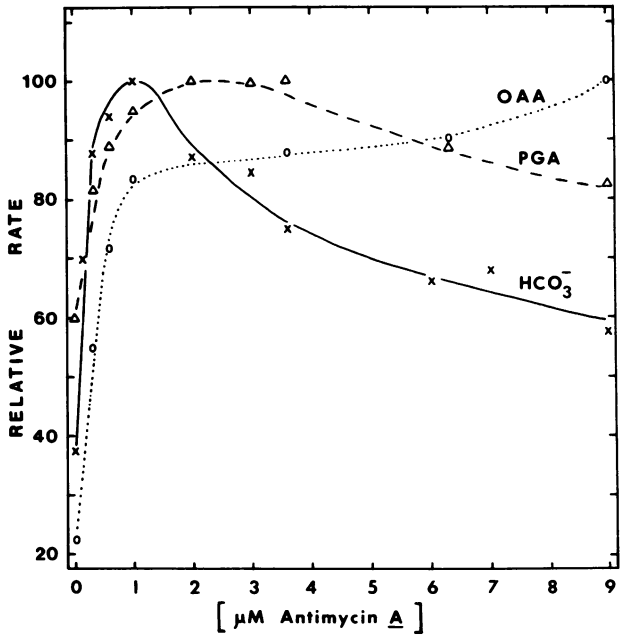

FIG. 3. Effect of antimycin A concentrations on anaerobic oxygen evolution with various acceptors. Curves for OAA (O···O), PGA (Δ- - -Δ), and HCO_3^- (×——×) were obtained in white light (5000 w m^{-2}) under initially anaerobic (<20 μM O_2) conditions. Control rates in μmol O_2/mg Chl·hr were 20, 65, 35 for OAA, PGA, and HCO_3^-, respectively.

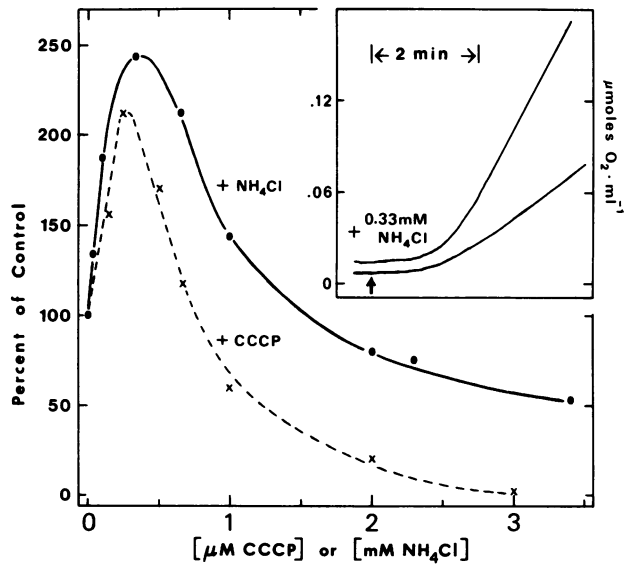

FIG. 4. Effect of uncouplers on anaerobic O_2 evolution with HCO_3^-. Curves were obtained with NH_4Cl (●——●) and CCCP (×- - -×). Reaction conditions and illumination were as in Figure 3 and under "Materials and Methods." Control rate of O_2 evolution without uncoupler was 44 μmol O_2/mg Chl·hr. Arrow at bottom of O_2 evolution traces in the inset indicates onset of illumination.

differential effects of methylamine and CCCP on PGA and bicarbonate reduction.

In Figure 5, the effects of both antimycin A and ammonium chloride on anaerobic O_2 evolution are presented as time courses for each of the electron acceptors studied. If samples are made anaerobic initially, the maximum steady-state rate with bicarbonate is lower than the aerobic rate and is enhanceable by addition of either antimycin A or ammonium chloride. Similarly, the rates with PGA and OAA are accelerated by these agents.

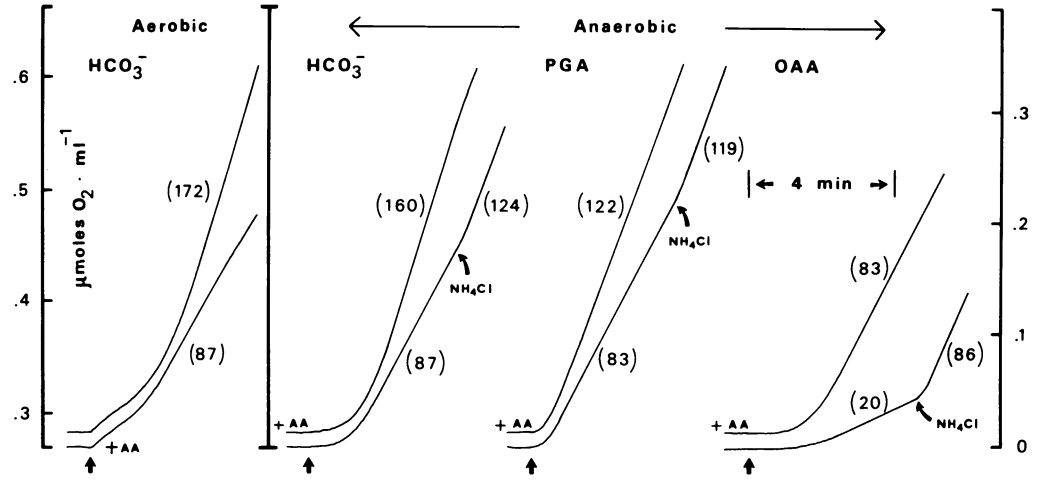

FIG. 5. O_2 evolution traces showing the response to antimycin A and uncoupler with different acceptors. Numbers in parentheses give the steady-state rates of O_2 evolution in µmol O_2/mg Chl·hr. Traces with 1 µM antimycin A present are labeled +AA. NH_4Cl additions were 0.6 mM in the case of HCO_3^- and PGA, and 3 mM in the case of OAA. Arrows at bottom of traces indicate light on. Values to the left of aerobic traces and to the right of anaerobic traces give the O_2 tension during the respective measurements. Aerobic samples were illuminated with 3,500 w m^{-2} of orange light and anaerobic samples with 5,000 w m^{-2} of white light. See Figure 3 and "Materials and Methods" for other reaction conditions.

It is improbable that an irreversible photoinhibition is the underlying cause for the low rates since they respond rapidly to the addition of uncoupler. From the traces it is evident that the anaerobic generation of NADPH (required for all three acceptors, OAA, PGA, and bicarbonate) is enhanced by either of two agents which have nothing in common save the ability, inferred in the case of antimycin A, to diminish the pH gradient across the thylakoid in the illuminated steady-state.

Table I shows the effects of ribose-5-P and antimycin A on bicarbonate-supported O_2 evolution at 50% of the maximum light intensity used in Figure 1. Although rate acceleration by antimycin A is still apparent, it is only 15% above the anaerobic control. The addition of ribose-5-P also accelerates the anaerobic rate and this could be taken as evidence that increased enzymic regeneration of the endogenous ribose-5-P pool is the source of the antimycin A effect. However, if this were the case then the addition of antimycin A to samples already containing ribose-5-P should have little or no effect, whereas the data in Table I show that both substances together give a rate lower than with either substance alone. A more feasible interpretation is that ribose-5-P, acting as an ATP sink (28), and antimycin A, acting as an inhibitor of cyclic electron transport, both serve to enhance the over-all CO_2 fixation rate by decreasing an excess phorphorylation potential. With both additives presented together, the ATP supply is decreased below a critical level and inhibition ensures just as is observed at superoptimal concentrations of antimycin A alone (Fig. 3) or ammonium chloride alone (Fig. 4).

The preceding interpretation of the stimulatory effect of antimycin A on anaerobic photosynthesis is supported by the radioactive tracer studies of Schacter *et al.* (22) and Schacter and Bassham (21). Under comparable conditions, they showed that, at steps in the carbon pathway requiring ATP, the reactant and product pool sizes change in a manner indicative of a decreased phosphorylation potential in the presence of antimycin A.

Aerobically, the control rates in Table I are higher than the anaerobic values whereas ribose-5-P are antimycin A significantly inhibit. Under aerobic conditions, the ATP/ADP ratio must already be properly regulated in relation to NADP reduction so that a decrease in the ratio, either by adding excess ribose-5-P or by inhibiting cyclic turnover, would give rise to the net inhibition of bicarbonate-dependent O_2 evolution also seen in Figure 1. In view of our near optimization of the conditions

TABLE I. Antimycin A and Ribose-5-P effects at intermediate light intensity.

Assay conditions as described in Materials and Methods. Intensity of orange light was 2500 w·m^{-2}. Additions to reaction medium containing chloroplasts (41 µg Chl·ml^{-1}) as indicated.

Additions	Rate of O_2 Evolution µmol/mg Chl·hr	Control %
Anaerobic (<20 µM O_2)		
+ HCO_3^- (15 mM)	130	100
" + Antimycin A (1 µM)	150	115
" + Rib-5-P (4.7 mM)	149	114
" + Antimycin A + Rib-5-P	133	102
Aerobic (290 µM O_2)		
+ HCO_3^-	168	100
" + Antimycin A	88	52
" + Rib-5-P	140	83

for bicarbonate reduction (*i.e.* relatively low Pi present) and the use of less than saturating light intensity, the inhibitory effects of ribose-5-P and antimycin A on the aerobic rate do not seem in contradiction to earlier work (27). In those studies, ribose-5-P or antimycin A increased the activity of chloroplasts inhibited by high Pi levels.

In Table II, results at intermediate light intensity with both antimycin A and ammonium chloride are presented according to the differing ATP requirements for each of the electron acceptors HCO_3^-, PGA, and OAA. A comparison of the controls with those containing antimycin A reveals that as the ATP/NADPH requirement is decreased, the acceleration of rates by antimycin A becomes larger. With the uncoupler, there is also an inverse relationship between the degree of acceleration and the requirement for ATP relative to NADPH. In the presence of both ammonium chloride and antimycin A, the over-all inhibition is most severe in the case of HCO_3^- which has the highest ATP/NADPH requirement of 3/2. The combination of antimycin A and uncoupler is less inhibitory to PGA reduction with an ATP/NADPH requirement of 1, whereas the uncoupled rate of OAA reduction is relatively unaffected by the further addition of antimycin A. From the table, it is clear that both the inhibitory

TABLE II. Summary of Antimycin A and uncoupler effects with various electron acceptors.

Assay conditions as described in Materials and Methods. Intensity of orange light was 2500 $w \cdot m^{-2}$. Additions to anaerobic reaction medium containing chloroplasts (45 μg Chl$\cdot ml^{-1}$) as indicated.

ATP/NADPH Requirement	Additions	Rate of O_2 Evolution $\mu mol/mg$ Chl$\cdot hr$	Control %
3/2	+ HCO_3^- (15 mM)	137	100
	" + Antimycin A (1 μM)	157	114
	" + NH_4Cl (3 mM)	72	52
	" + Antimycin A + NH_4Cl	16	11
1/1	+ PGA (6.5 mM)	74	100
	" + Antimycin A	94	127
	" + NH_4Cl	82	111
	" + Antimycin A + NH_4Cl	65	87
0/1	+ OAA (7 mM)	42	100
	" + Antimycin A	92	230
	" + NH_4Cl	85	202
	" + Antimycin A + NH_4Cl	86	204

and stimulatory effects of antimycin A and uncouplers are related to the generation of ATP and the ability of various electron acceptors to utilize ATP.

CONCLUSIONS

The main conclusions to be drawn from this study of intact chloroplasts can be summarized as follows.

A. The rates of CO_2 fixation at high light and bicarbonate levels are depressed under anaerobic conditions.

B. Antimycin A prevents the inhibitory effect of anaerobiosis.

C. The uncoupling agents CCCP and ammonium chloride also prevent the inhibitory effect of anaerobiosis.

D. The reductions of terminal electron acceptors which require only a few or none of the Calvin cycle enzymes, *e.g.* PGA and OAA, respectively, are also accelerated by antimycin A under anaerobic conditions.

E. The acceleration by antimycin A is greater and extends to lower light intensities as the demand for ATP in the metabolism of the acceptor decreases.

F. Antimycin A inhibition of aerobic CO_2 fixation suggests a need for cyclic activity *in vivo*.

While there is no conclusive evidence in this study to differentiate between the phenomenological similarities produced by antimycin A and uncouplers, their primary mechanisms of action would appear to differ on the basis of the following considerations: cyclic electron transport in plants is thought to involve a *b* type Cyt as does the cyclic system in photosynthetic bacteria and the respiratory chain in mitochondria. In the latter two cases, antimycin A has been shown to block the electron transfer between the *b* and *c* type Cyt (19, 23). Furthermore, data in the chloroplast literature (3, 8) show no evidence for uncoupling at the low concentrations of antimycin A employed here to overcome the anaerobic inhibition of linear electron transport. Although cyclic electron transport processes are sensitive to antimycin A in the region of 0.1 to 5 μM (3, 8, 15) and an inhibition of linear electron flow occurs in the range of 1 to 10 μM (8), uncoupling activity is only apparent at concentrations exceeding 10 μM (8, 13).

It one assumes that Cyt *b*-563 as an integral component of coupled cyclic electron transport, the inhibition by antimycin A at the level of Cyt *b*-563 would be consistent with the observations reported here. Spectroscopic experiments utilizing antimycin A to estimate the relative contributions of linear and cyclic electron flow to photophosphorylation are in progress.

This investigation does not claim to disprove the concept of indirect enzyme activation by antimycin A (7, 18, 21, 22, 28). Indeed, the increased throughput of electrons to ferredoxin in response to antimycin A or uncouplers could result in a change of its redox status with subsequent effects on enzymes which are known to be light-regulated. It remains to be established under what conditions control of electron flow by the proton gradient and control of carbon cycle enzyme activities might contribute to the over-all regulation of photosynthesis.

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