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CYCLIC ELECTRON TRANSPORT IN ISOLATED INTACT CHLOROPLASTS

FURTHER STUDIES WITH ANTIMYCIN

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Summary

Antimycin has been used to study the role of cyclic electron transport in isolated intact chloroplasts maintained under aerobic conditions.

At all light intensities, antimycin inhibits CO_2 fixation when assay conditions are optimal. When turnover of the Calvin cycle is inhibited, antimycin stimulates bicarbonate-dependent O_2 evolution. Energy-dependent processes such as chlorophyll *a* and 9-aminoacridine fluorescence quenching, and light-scattering (apparent absorption) changes are inhibited by antimycin. The results suggest that cyclic electron transport contributes to photophosphorylation under aerobic conditions and is obligatory as a source of ATP during the most active periods of CO_2 fixation in vivo.

Cyclic electron transport can be stimulated either by inhibiting Photosystem II activity or increasing the turnover of Photosystem I relative to Photosystem II. These effects are interpreted in terms of the need for correct redox poising of carriers in the pathway in order to sustain maximum rates of cyclic electron flow.

Binding studies indicate the presence of a high affinity antimycin binding site on chloroplast membranes. The stoichiometry and dissociation constant of the high affinity site are consistent with the idea that antimycin inhibits cyclic electron transport by binding to a *b*-type cytochrome in the thylakoid membrane.

Introduction

Photosynthetic reduction of CO_2 to the level of sugar phosphates by the enzymes of the Calvin cycle requires ATP and NADPH in the mol ratio 3 : 2.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine.

However this ratio [1] may be considerably higher if further metabolism (e.g., to starch [2]) or prior fixation (e.g., by the C4 pathway [3]) occurs. There are varying opinions as to whether photophosphorylation coupled to reduction of $NADP^+$ by non-cyclic electron transport can supply enough ATP to meet these requirements [4–6]. If non-cyclic electron transport cannot supply sufficient ATP, then the deficiency may be offset by photophosphorylation associated with either pseudocyclic electron flow [7–9] (reduction of O_2 by a coupled segment of the linear electron transport chain, also known as Mehler reaction), or by ferredoxin-mediated cyclic electron flow around Photosystem I [8,10–14].

Recent studies have shown that isolated intact chloroplasts capable of high rates of CO_2 fixation can also sustain relatively high rates of concurrent pseudocyclic electron flow [15,16]. On the basis of the high apparent affinity of the unknown terminal electron transport component for O_2, it has been proposed that coupled pseudocyclic rather than cyclic electron flow is initiated during periods of ATP deficiency [16,17]. On the other hand, isolated intact chloroplasts are capable of maintaining high rates of cyclic electron flow under air [18] or deoxygenated conditions [19,20] when non-cyclic electron transport is inhibited. The question whether or not cyclic electron transport occurs at high rates during CO_2 fixation is still unresolved.

This problem has been studied using antimycin, an inhibitor of ferredoxin-catalysed cyclic photophosphorylation [10,14]. Several groups of workers have studied the effect of antimycin on CO_2 fixation in isolated chloroplasts [10–13,19–27] with conflicting results. Both an inhibition [19,20,23] and a stimulation [21,22,24–26] of rate have been observed. However, the stimulation by antimycin invariably occurred when the initial rate of CO_2 fixation was suboptimal, either because of the presence of inhibitory levels of inorganic phosphate [26], omission of ascorbate during chloroplast isolation [21,22,24, 25], or the use of anaerobic conditions and high light intensity [21,22,27]. In this communication we will show that under optimal assay conditions, antimycin inhibits both the rate of CO_2 fixation and other energy-dependent processes such as slow quenching of chlorophyll *a* fluorescence. The results indicate that turnover of cyclic electron transport is obligatory to maintain the highest rates of CO_2 fixation. The activity of non-cyclic electron transport also may regulate cyclic turnover through 'poising' of carriers common to the two pathways.

Materials and Methods

Intact chloroplasts were isolated from spinach (*Spinacia oleracea*) as described previously [27]. Chloroplasts were greater than 70% intact as judged by the ferricyanide method [28].

Chlorophyll *a* fluorescence was excited by a weak (0.5 W/m^2) measuring beam obtained by filtering the output of a mercury lamp through Corning 0-53, 4-96 and Balzers DT Blau filters. The beam was modulated at 270 Hz and modulated fluorescence (around 685 nm) detected by a Princeton Applied Research model 120 lock-in amplifier after transmission through a Bausch and Lomb monochromator blocked with a Corning 2-58 cutoff filter. Blue actinic

light (100 W/m^2) was provided by a 500 W projector and Corning 4-76 and 4-96 filters. 9-Aminoacridine fluorescence was measured on the same apparatus with the following filter combinations: measuring beam, Corning 7-39; photomultiplier, Corning 4-96 and monochromator at 460 nm; actinic light (100 W/m^2), Corning 2-58 filter. Light-induced changes in apparent absorption were measured at 518 nm using a single beam spectrophotometer with vertical optics. Actinic illumination (100 W/m^2) was similar to that provided for 9-aminoacridine measurements. O_2 evolution was measured with a Clark-type oxygen electrode [27] with illumination conditions similar to those used in chlorophyll *a* or 9-aminoacridine fluorescence experiments.

For all above experiments, chloroplasts were suspended at 18—22 μg chlorophyll/ml (determined by the method of Arnon [29]) in a medium consisting of 0.35 M sorbitol/0.25 mM Na_2HPO_4/50 mM Tricine brought to pH 8.15 with 10 M NaOH and KOH.

Antimycin binding experiments were carried out by assaying the concentration of antimycin remaining in solution after chloroplasts were removed by centrifugation. Antimycin concentration was determined by its fluorescence detected with a Perkin-Elmer MPF4 spectrofluorimeter. Calibration was determined by adding antimycin to chloroplast supernatant after chloroplast removal. The chlorophyll concentration used in these experiments is indicated in the figure legend.

Results

Involvement of cyclic electron transport in CO_2 fixation

Table I shows the effect of 1 μM antimycin on the rate of CO_2-dependent O_2 evolution by isolated spinach chloroplasts. Either an inhibition or a stimulation of the rate of CO_2 fixation was observed depending on the assay conditions. Under optimal conditions giving the highest rates of CO_2 fixation, antimycin was invariably inhibitory. A previous report from this laboratory showed that rates of CO_2-dependent O_2 evolution in excess of 100 μmol/mg chlorophyll per h could be obtained under high intensity (500 W/m^2) illumination and that antimycin was inhibitory under these conditions [27]. Table I extends these observations to intermediate (100 W/m^2) and low (20 W/m^2) light intensities

TABLE I

EFFECT OF ANTIMYCIN ON CO_2 FIXATION IN ISOLATED SPINACH CHLOROPLASTS

Control consisted of chloroplasts (21 μg chlorophyll/ml) suspended in reaction medium (see Materials and Methods) which also contained 8.3 mM $NaHCO_3$ and catalase, 1500 units/ml. Light intensity was 110 W/m^2.

Treatment	Rate of CO_2 fixation (μmol O_2 evolved/mg chlorophyll per h)	
	No antimycin	Plus 1.0 μM antimycin
Control	66	32
Low light (20 W/m^{-2})	10	5
Minus catalase	12	37
Plus 0.9 mM P_i	10	33

and shows that an antimycin-sensitive reaction is required to observe the highest rates of CO_2 fixation under all intensities of illumination. Omission of catalase, or the presence of relatively high levels of inorganic phosphate both result in depressed rates of CO_2 fixation, which have been explained on the basis of depletion of Calvin cycle intermediates [26,30]. Under these conditions, antimycin stimulated the depressed rate of CO_2-dependent O_2 evolution (Table I). However the stimulated rate was not significantly higher than rates observed with control chloroplasts treated with antimycin. These observations reconcile our results with those of others and suggest that earlier reports of antimycin stimulation of CO_2 fixation resulted from the use of inhibitory conditions to assay CO_2 fixation [21,22,24–26].

The effects of antimycin on CO_2 fixation may be due to inhibition of cyclic electron transport by this antibiotic [10–14]. To investigate this possibility, we have studied the slow quenching of chlorophyll *a* fluorescence that occurs on illumination of intact chloroplasts [31–33]. Slow fluorescence quenching is known to depend on the development and maintenance of the proton gradient (or high energy state) across the thylakoid membrane. The quenching probably results from membrane conformational changes that occur as protons taken up in the light become bound to the inner surface of the thylakoid [31,32,34]. Since proton pumping is coupled to both cyclic and non-cyclic electron flow, slow quenching of fluorescence may conveniently be used to monitor the activity of both types of electron transport in the intact organelle. As shown in Fig. 1 and reported earlier by Barber and coworkers [31,34] considerable slow quenching of fluorescence is observed even under conditions when O_2 is the

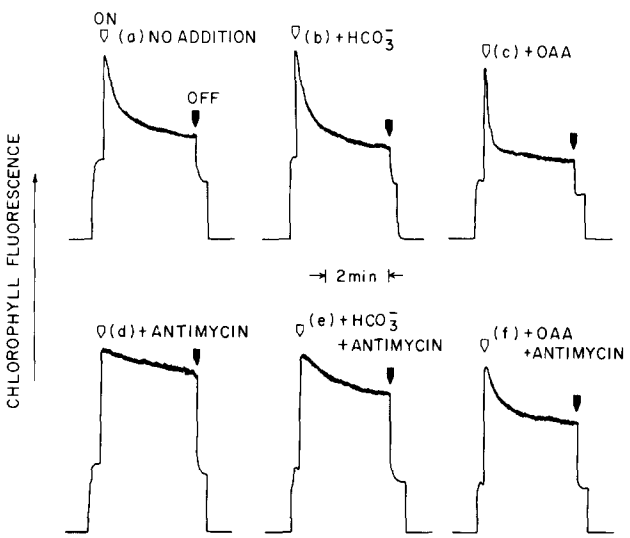

Fig. 1. Effect of 1.0 μM antimycin on slow chlorophyll *a* fluorescence quenching in isolated intact chloroplasts. The modulated measuring beam was sufficiently intense to cause some reduction (approx. 25%) of Q, the primary electron acceptor of Photosystem II. The rapid responses to the unmodulated actinic beam represent redox changes of the remaining Q pool. Bicarbonate and oxaloacetate concentrations were 8.3 and 1.67 mM, respectively. The kinetics of slow quenching depend to some extent on the history of the sample. To minimise this effect, each measurement was performed on a fresh, dark-adapted sample.

only available terminal electron acceptor for non-cyclic electron transport. This quenching has been attributed to proton pumping associated with endogenous pseudocyclic electron flow [33,34]. However, 1 μM antimycin almost completely inhibited the quenching (Fig. 1d). Addition of oxaloacetate, which requires NADPH (but not ATP) for its reduction greatly increases the rate of slow quenching while only slightly increasing the extent of quenching [31,34]. Bicarbonate can partially bring about the same effects as oxaloacetate. The results have been explained in terms of increased rates of coupled non-cyclic electron flow and proton pumping in the presence of these electron acceptors [31—35]. Again however, Fig. 1 shows that antimycin greatly inhibited slow quenching even in the presence of oxaloacetate and CO_2. In the presence of bicarbonate, antimycin was observed to inhibit quenching under conditions where oxygen evolution was either inhibited or stimulated (as in Table I). These effects of antimycin are in some ways similar to those of an uncoupler, but at the low concentrations used here, antimycin does not act as a classical uncoupler of ferricyanide or methyl viologen-dependent photophosphorylation in broken chloroplasts [36]. The action of antimycin on CO_2-dependent oxygen evolution and chlorophyll fluorescence also does not result from inhibition of non-cyclic electron flow, for Table II shows that uncoupled rates of NADP-dependent oxaloacetate reduction are little affected by antimycin up to a concentration of 10 μM. Table II also shows that low concentrations (less than 0.33 μm) of antimycin stimulated coupled oxaloacetate reduction up to the uncoupled rate therefore antimycin inhibition of slow quenching cannot be related to an increased reduction of Q (the primary electron acceptor of Photosystem II) since in the presence of oxaloacetate, Q would be more oxidized. The results suggest that under all conditions thus far discussed, coupled cyclic electron transport occurs at significant rates in illuminated intact chloroplasts and it is this process which is inhibited by antimycin.

This conclusion is qualitatively supported by studies of 9-aminoacridine fluorescence quenching by illuminated intact chloroplasts. Quenching of 9-aminoacridine fluorescence has been related to the magnitude of the light-induced pH gradient developed across the thylakoid membrane [37]. Quantitative interpretation of such experiments using intact chloroplasts must, however,

TABLE II

EFFECT OF ANTIMYCIN ON OXALOACETATE REDUCTION IN COUPLED AND UNCOUPLED SPINACH CHLOROPLASTS

Conditions as in Table I except medium contained 5 mM oxaloacetate in place of bicarbonate.

Antimycin (μM)	Rate of oxaloacetate reduction (μmol O_2 evolved/mg chlorophyll per h)	
	No NH_4Cl	Plus 3.3 mM NH_4Cl
None	30	44
0.33	45	45
1.0	43	42
2.0	39	39
5.0	40	40
10.0	40	40

be made with care (see discussion). Table III shows that considerable quenching of 9-aminoacridine fluorescence does occur on illumination of intact organelles. The quenching is stimulated by oxaloacetate (but not bicarbonate) and is almost completely abolished by monensin (which facilitates Na^+-H^+ exchange across thylakoids). As expected, antimycin inhibits the quenching under all conditions, but this inhibition is much smaller than that of chlorophyll fluorescence quenching and is less affected by bicarbonate or oxaloacetate.

Poising of cyclic electron flow

It has now become apparent that optimal conditions for cyclic electron transport activity include correct redox 'poising' of the carriers in the pathway [12—14,18,19]. The inhibitor of non-cyclic electron transport, DCMU, has often been shown to stimulate cyclic electron transport presumably by preventing 'overreduction' by Photosystem II of key electron carriers common to both cyclic and non-cyclic pathways [10,12—14,18].

A similar stimulatory effect of DCMU on cyclic electron transport, as monitored by chlorophyll *a* fluorescence quenching, can be observed. Fig. 2 shows that in the presence of a low concentration of DCMU (0.1 μM), the rate of fluorescence quenching was greatly increased (see Fig. 1) independent of the presence or absence of bicarbonate. The quenching was very sensitive to inhibition by antimycin. Since this concentration of DCMU greatly inhibits non-cyclic electron transport (as seen by the considerable reduction of Q elicited by the measuring beam), the increased rate of quenching can only be ascribed to an increased rate of cyclic electron transport.

The final steady state of quenching depends on the concentration of DCMU and the presence of bicarbonate. As shown in Fig. 3, the extent of quenching is not maximal in the absence of bicarbonate but is stimulated to the maximum by adding DCMU up to a concentration of 0.1 μM. This concentration of DCMU presumably optimally poises the cycle by preventing overreduction of cycle intermediates. Above 0.1 μM, chlorophyll fluorescence quenching becomes inhibited presumably because now the cycle carriers are 'overoxidised'. In the presence of bicarbonate, the extent of quenching is already maximal and is more sensitive to inhibition on increasing the concentration of DCMU. These latter results are predictable if bicarbonate stimulates the quenching by increasing non-cyclic electron flow. On the other hand, the great sensitivity of quenching to antimycin suggests that bicarbonate might indirectly

TABLE III

EFFECT OF ANTIMYCIN ON 9-AMINOACRIDINE QUENCHING OBSERVED IN ISOLATED INTACT CHLOROPLASTS

Addition	Percent of 9-aminoacridine fluorescence quenched	
	No antimycin	Plus 1.0 μM antimycin
None	50	43
8.3 mM HCO_3^-	48	42
1.67 mM oxaloacetate	64	54
0.5 μM monensin	2	—

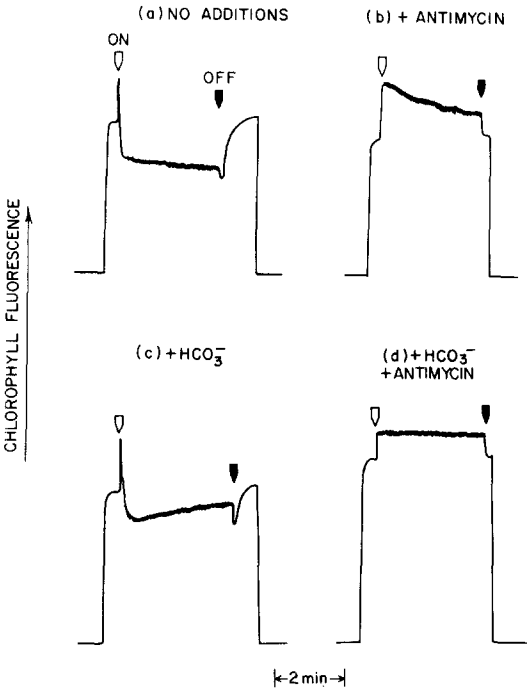

Fig. 2. Effect of 1.0 μM antimycin on slow chlorophyll fluorescence changes in the presence of 0.1 μM DCMU. Bicarbonate concentration was 8.3 mM.

affect cyclic electron transport. If bicarbonate oxidizes the cyclic carriers through the reoxidation of NADPH, then stimulation of cyclic electron transport would occur when the cycle is normally overreduced (—DCMU). Likewise the oxidizing effect of bicarbonate would be inhibitory when the cycle is optimally poised or overoxidised. This would explain the effect of bicarbonate

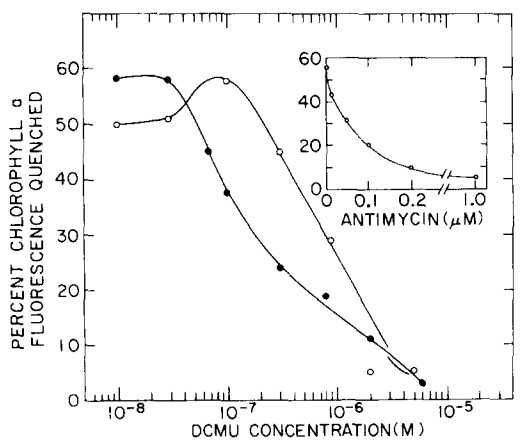

Fig. 3. Effect of DCMU on slow chlorophyll *a* quenching in the presence of (solid circles) and absence (open circles) of 8.3 mM bicarbonate. Insert shows antimycin inhibition of maximal quenching obtained (no bicarbonate, 0.1 μM DCMU).

on the DCMU inhibition curve (Fig. 3). Furthermore, the antagonistic effect of Photosystem II and the Calvin cycle on the redox state of Photosystem I might give rise to biphasic kinetics of the type shown in Fig. 2c.

The results presented above suggest that the rate of non-cyclic electron transport might influence cyclic electron transport through the redox state of electron carriers common to both pathways. This idea is supported by the results shown in Fig. 4 which depicts the effect of oxaloacetate and antimycin on energy-dependent membrane structural changes which can be observed as a slow increase in apparent absorption at 518 nm [38]. Clearly the slow 518 nm response is small in the absence of electron acceptors but is increased by adding oxaloacetate which stimulates non-cyclic electron transport. However, the slow structural change is greatly inhibited by antimycin indicating that it is mainly energized by the activity of cyclic rather than non-cyclic electron flow. It seems clear in this case that oxaloacetate-stimulated non-cyclic electron flow can also activate coupled cyclic electron transport.

Stoichiometry of antimycin binding to the chloroplast

Antimycin possesses a well defined and relatively intense fluorescence which can be used to assay its concentration in dilute solutions [39,40]. We have utilised this property to study the binding of antimycin to intact chloroplasts. Fig. 5a shows that under the conditions used so far, chloroplasts take up a constant proportion of the antimycin initially added to the medium. At a higher chloroplast concentration (133 μg chlorophyll/ml) very little antimycin remains 'free' in solution after removal of membranes by centrifugation. The insert of Fig. 5a shows that antimycin uptake by chloroplasts is a linear function of the concentration of inhibitor added. The results are consistent with a high partitioning of antimycin from the medium to the lipid phase of the chloroplast rather than any specific binding to high affinity sites. In order to detect any high affinity sites present in low concentrations, it is necessary to

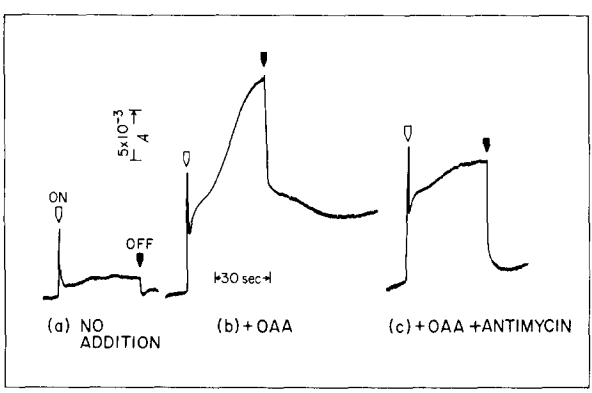

Fig. 4. Effect of oxaloacetate on slow changes in apparent absorption at 518 nm. Samples were flushed with N_2 to reduce the O_2 content as described previously [26]. Oxaloacetate concentration was 3.0 mM and antimycin was 4.5 μM. Note that oxaloacetate stimulates the slow *P*-518 increase and this is greatly inhibited by antimycin. Oxaloacetate also stimulates the rapid *P*-518 response (which may reflect a membrane potential) but the rapid phase is not sensitive to antimycin. In all cases, measurements were taken after several light/dark cycles when the absorption response was completely reversible and reproducible.

increase the concentration of chloroplasts and decrease non-specific antimycin uptake. This can be achieved by including bovine serum albumin in the medium. Albumin has been shown to bind antimycin reversibly with an apparent dissociation constant of 0.12 μM [39]. In the presence of albumin, non-specific antimycin uptake is greatly reduced (Fig. 5b) as is the case with mitochondrial particles [39,40].

Fig. 5b shows that in the presence of albumin, low concentratons of antimycin become specifically bound to chloroplasts. Since some non-specific uptake persists under these conditions, apparent binding of antimycin to chloroplasts is a biphasic function of inhibitor added (insert, Fig. 5b). This fact, together with the binding to albumin itself, makes estimation of the kinetic parameters of the chloroplast high affinity site difficult. For example, a Scatchard plot of the data in Fig. 5b shows considerable curvature due to non-specific antimycin uptake (results not shown). However one can estimate that the number of sites is approx. 1.4 nmol/mg chlorophyll (or 1 mol per 800 mol

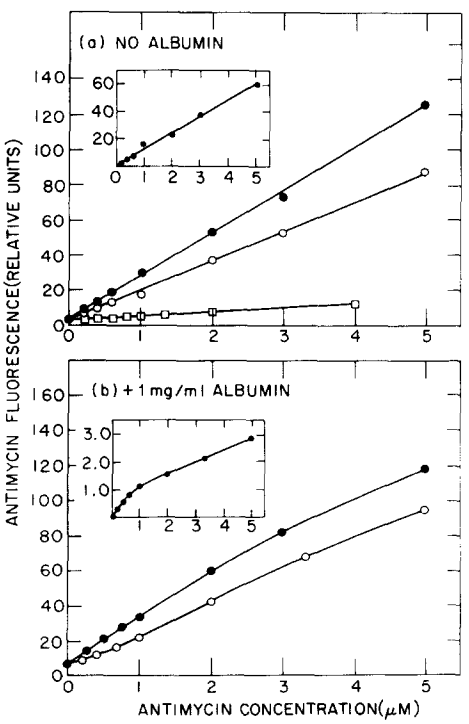

Fig. 5. Binding of antimycin to chloroplasts. Experiments were performed as follows. Antimycin at concentrations indicated by the horizontal axis was added to chloroplasts and incubated in a shaking water bath for 20 min. The chloroplasts and any bound antimycin were removed by centrifugation and the fluorescence of antimycin remaining in solution recorded (vertical axis). Control curves (•) were performed by incubating chloroplasts without antimycin, spinning down the membranes, then adding antimycin to the supernatant. The difference between the experimental and control curves represents antimycin bound to chloroplasts during incubation. From this difference, the antimycin bound to the chloroplast (in nmol/mg chlorophyll) can be calculated and this is shown in the insert (vertical axis) as a function of antimycin concentration originally added. (a) No albumin present. ○, chloroplasts: 27 μg chlorophyll/ml; □, chloroplasts: 133 μg chlorophyll/ml. (b) In the presence of 1 mg/ml albumin. Chloroplasts were 460 μg chlorophyll/ml.

chlorophyll). The apparent dissociation constant is approx. $5 \cdot 10^{-7}$ M which, when corrected for binding to albumin yields a 'true' dissociation constant (K_d) of approx. $4 \cdot 10^{-9}$ M. If this K_d is indeed correct, then higher concentrations of albumin should succesfully compete with the chloroplast sites for antimycin and thereby reduce the high affinity binding observed in Fig. 5b. We did in fact see no antimycin binding to chloroplasts in the presence of 5 mg/ml albumin in agreement with this prediction. At the latter concentration of albumin (approx. 70 μM), the concentration of 'free' antimycin is reduced to less than 1.0 nM. This result provides confidence that the K_d value of $4 \cdot 10^{-9}$ M for the chloroplast high affinity site is correct to within an order of magnitude. The above estimate of the number of high affinity sites (n) should be regarded as a lower limit, since it is by no means certain if they were fully saturated under the conditions used. However, it is also possible to estimate an upper limit for n from the data of Fig. 5a. Since no specific binding was detectable in the latter case at the lowest concentration of antimycin used, an upper limit of n = 7.5 nmol/mg chlorophyll (1 mol per 150 mol chlorophyll) can be inferred.

Discussion

It has been clearly shown in this paper that antimycin inhibits aerobic CO_2 fixation under optimal conditions, but stimulates the rate under sub-optimal assay conditions. It seems likely that both these effects are due to an inhibition by antimycin of cyclic electron transport in the intact organelle. At the concentration used, antimycin has been shown to be a potent inhibitor of cyclic phosphorylation in broken chloroplasts [10—14]. It has also been concluded that antimycin exerts a similar effect in intact chloroplasts as judged directly by measurements of adenine nucleotide pools [24,25,36] and indirectly from changes in Calvin cycle intermediates [22—25]. On this basis, inhibition by antimycin of maximal rates of CO_2 fixation are simply ascribed to the decreased supply of ATP normally provided by cyclic phosphorylation. However, it is harder to explain why under some circumstances (no catalase present, inhibitory phosphate), such a diminution in ATP should stimulate an inhibited rate of CO_2 fixation. It is pertinent that under the conditions where antimycin has a stimulatory effect, CO_2 fixation is already limited not by ATP but by decreased turnover of the Calvin cycle probably caused by depletion of cycle intermediates [26,30]. It is possible that decreased utilisation of ATP causes further inhibition of non-cyclic electron transport by the resulting back pressure of the proton gradient. Antimycin inhibition of coupled cyclic electron transport would decrease the high energy state back pressure and stimulate non-cyclic electron flow. Such a mechanism was proposed to explain similar effects of antimycin under anaerobic conditions and high light intensity [27] but the proposal was not supported by subsequent experiments [36]. An alternative explanation is that CO_2 fixation is particularly sensitive to changes in enzyme activity when the Calvin cycle is rate limiting. It is possible that antimycin may increase the pool of reduced ferredoxin by inhibiting cyclic electron flow and thereby activate certain key enzymes via the recently documented ferredoxin/thioredoxin system [41,42]. Experiments are currently in progress to test these possibilities.

Although it is stressed here that cyclic electron transport occurs under aerobic conditions, it has previously been concluded that O_2 inhibits cyclic electron flow in vivo [17]. In fact, experiments reported elsewhere [36] show that in isolated chloroplasts, O_2 stimulates both pseudocyclic and cyclic electron flow. This effect appears to be related to the need for correct 'poising' of the redox carriers in the cyclic electron transport pathway. In the absence of O_2 and an acceptor of non-cyclic electron flow, Photosystem II activity causes key electron transport carriers common to both cyclic and non-cyclic pathways to be 'overreduced'. Treatment such as addition of O_2 or oxaloacetate, (and to some extent HCO_3^-) to restore turnover of Photosystem I, or addition of DCMU to inhibit photosystem II, will increase cyclic electron transport. This presumably results from partial oxidation of a key carrier which becomes better poised for the operation of cyclic electron transport under these conditions.

It therefore seems likely that the relative activity of both non-cyclic and pseudocyclic electron flow may partially determine the rate of cyclic turnover under aerobic conditions. Presently, it is difficult to evaluate this idea because of the problems of quantitatively estimating the contribution of cyclic electron flow to phosphorylation when both pseudocyclic and non-cyclic electron flows occur together with ATP-consuming reactions such as CO_2 fixation. Measurements of O_2 evolution, chlorophyll *a* fluorescence and apparent absorption changes at 518 nm all suggest that cyclic activity contributes considerably to the high energy state under conditions where non-cyclic electron flow rates are high (see also ref. 35). Experiments with 9-aminoacridine revealed only small effects of antimycin suggesting that cyclic electron flow contributes little to the transthylakoid pH gradient. However, it is worth pointing out here that 9-aminoacridine fluorescence may not be an ideal probe for ΔpH in the intact organelle. 9-Aminoacridine quenching is presumed to result from its accumulation in the intrathylakoid space in response to the lowered intrathylakoid pH in the light [37]. However, according to Heldt and colleagues [43,44], the in vivo pH gradient is contributed in part by alkalinisation of the stroma. As pointed out also by Tillberg et al. [45], 9-aminoacridine fluorescence would not respond to a rise in stromal pH but would signal the difference in pH between the thylakoid and the outside suspending medium. Based on this idea and using the data of Werdan et al. [44] with the equations of Schuldiner et al. [37] it is easy to calculate that quenching of 9-aminoacridine in intact chloroplasts in the presence of bicarbonate should be approx. 1% when the pH of the medium is 7.6 and approx. 3% when the external pH is 8.15. However the experimentally observed quenching under these conditions is much larger than that calculated 20—40% at pH 7.6 [45] and nearly 50% at pH 8.15). Clearly, therefore, pH as measured by Heldt and colleagues using [^{14}C]methylamine does not agree with that using 9-aminoacridine. Until the discrepancies are adequately accounted for, the quantitative aspects of both methods should be regarded with caution.

The inhibitory action of antimycin on mitochondrial electron transport has been related to high affinity binding of the inhibitor to *b*-type cytochromes [39,40]. We have demonstrated that chloroplasts also possess a high affinity binding site for antimycin which exists at approximately the same concentra-

tion (1 mol per 150—800 mol chlorophyll) as do *b*-type cytochromes in intact organelles [46]. We are currently investigating the possibility that antimycin inhibition of cyclic electron flow is also brought about through its interaction with a *b*-type cytochrome.

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