Inhibition of Photosynthetic Reactions by Aureomycin

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The effect of aureomycin on photosynthesis was investigated. This antibiotic which has been reported to stimulate photosynthesis at very low concentrations is an effective inhibitor at higher concentrations. In mesophyll protoplasts and isolated chloroplasts from spinach, 50% inhibition of CO_2 reduction required about 20 μM aureomycin. The reduction of 3-phosphoglycerate and of oxaloacetate by intact chloroplasts was also inhibited, but not that of nitrite and methylviologen which was actually stimulated. NADP reduction by broken chloroplasts and methylviologen-dependent photophosphorylation were also sensitive to aureomycin. The electrochromic shift at 518 nm which indicates formation of a light-dependent membrane potential was suppressed in the presence of 200 μM aureomycin and the transthylakoid proton gradient was decreased. The data confirm reports that aureomycin has uncoupling properties, and they indicate that it also acts as an inhibitor of ferredoxin/NADP reductase.

Introduction

Tetracyclines are widely used in chemotherapy. They prevent translation of genetic information by interacting with bacterial ribosomes thereby inhibiting bacterial protein synthesis [1]. Medical application is wide-spread because of relative non-toxicity which suggests high specificity of action. As a feed supplement, aureomycin, a representative of the group of tetracyclines, has long been known to stimulate growth of animals [2]. Aureomycin chelates cations [3], and it has been reported to have uncoupling properties [4]. Like other uncoupling amines [5–8], it may fail to inhibit photosynthesis of chloroplasts and can even enhance photophosphorylation of thylakoid membranes at low concentrations [9]. Stimulation of leaf photosynthesis has also been reported [10]. In this publication, we demonstrate interaction of aureomycin with chloroplast membranes (see also ref. [11]). Apparently, specificity of aureomycin action is limited. Aureomycin may be used as a tool in studying chloroplast bioenergetics and electron transport.

Materials and Methods

Mesophyll protoplasts were isolated from freshly harvested spinach leaves and purified as described by Giersch *et al.* [12]. Intact chloroplasts exhibiting rates of CO_2 assimilation between 100 and 160 μmoles per mg chlorophyll per hour were isolated from spinach according to Jensen and Bassham [13]. Ferricyanide reduction indicated that between 80 and 90% of the chloroplasts had intact envelopes in different chloroplast preparations [14]. Light-dependent oxygen evolution in the presence of different substrates was measured in a Clark type electrode or in the electrode described by Delieu and Walker [15]. The assay medium contained 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylene diamine tetraacetate, 10 mM NaCl, 0.5 mM KH_2PO_4, 50 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonate), pH 7.6, and 1000 units/ml catalase. Electron acceptors were $NaHCO_3$, 3-phosphoglycerate, oxaloacetate, $NaNO_2$ (usually 2 mM) or 10 μM methylviologen. For reduction of the latter compound, 2 mM KCN was also added. Reduction of substrate amounts of NADP by chloroplasts osmotically shocked in water or 10 mM $MgCl_2$ was recorded during illumination with red light at 340 nm in a crossbeam system. Photophosphorylation of ruptured chloroplasts was measured with a pH electrode according to Dilley [16]. Fluorescence of 5 μM 9-aminoacridine in a suspension of intact chloroplasts [5] was excited by a weak 415 nm beam, collected by fiber optics and recorded at 488 nm by a photomultiplier. Chlorophyll fluorescence was recorded at 740 nm. The actinic light was filtered through K 65 and Calflex C filters (from Balzers, Liechtenstein) and a RG 610 filter (from Schott, Mainz). It had a half band width from 626 to

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675 nm. The detectors were protected against actinic light by suitable filter combinations. For measurements of ATP, ADP or NADP in intact chloroplasts, $HClO_4$ was added to darkened or illuminated chloroplast suspensions to a final concentration of 0.7 M. After 5 min standing at room temperature, insoluble material was sedimented and the supernatant neutralized with K_2CO_3. Adenylates were measured by luminescence as in ref. [17] and NADP by enzymic cycling [18]. Ferredoxin-NADP reductase was assayed by recording the reduction of ferricyanide by NADPH at 420 nm [19].

Results and Discussion

CO_2-dependent oxygen evolution by mesophyll protoplasts or by intact chloroplasts was found to be sensitive to aureomycin. Appreciable stimulation of photosynthesis by very low aureomycin concentrations [10] was not seen in our experiments, and higher concentrations were clearly inhibitory. Inhibition did not become immediately effective. Usually about 2 min were required to develop inhibition. In isolated chloroplasts, about 20 μM aureomycin produced 50% inhibition, and full inhibition was observed at aureomycin concentrations above 50 μM (Fig. 1). Chloroplast reactions similarly sensitive to aureomycin as CO_2 assimilation were phosphoglycerate-dependent and oxaloacetate-dependent oxygen evolution. However, reduction of

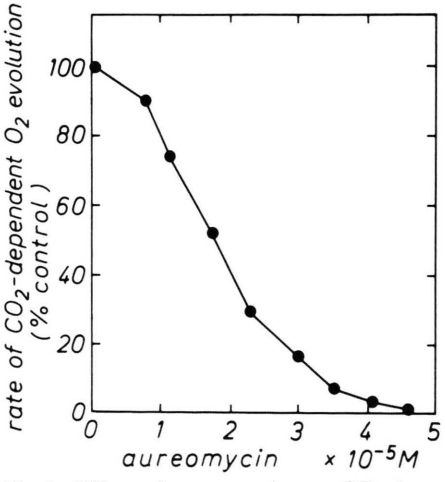

Fig. 1. Effect of aureomycin on CO_2-dependent oxygen evolution by intact chloroplasts. The control rate (100%) was 91 μmol O_2 evolution/mg chlorophyll per hour.

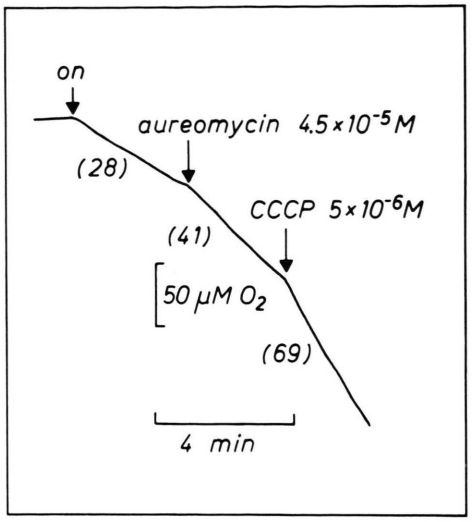

Fig. 2. Stimulation of methylviologen-dependent oxygen uptake by aureomycin and the uncoupler CCCP as recorded by an oxygen electrode. Numbers in brackets give rates of O_2 uptake in μmoles/mg chlorophyll per hour. "On" denotes onset of illumination with rate-saturating light.

methylviologen by intact chloroplasts was not only not inhibited by 45 μM aureomycin but actually stimulated suggesting some uncoupling (Fig. 2). Stimulation was increased by carbonyl cyanide-m-chlorophenylhydrazone (CCCP) which is a very potent protonophore. Enhancement of methylviologen reduction is difficult to reconcile with inhibition of oxaloacetate reduction. In contrast to the reduction of CO_2 or 3-phosphoglycerate, reduction of methylviologen and oxaloacetate does not require ATP and is under control of a high intrathylakoid proton concentration [17]. If the intrathylakoid pH is increased by NH_3 or CCCP, reduction of oxaloacetate or methylviologen is stimulated. Methylviologen reduction does not require ferredoxin and NADPH, while oxaloacetate is reduced by NADPH. The difference in aureomycin sensitivity of these reductive reactions suggested that either the reduction of ferredoxin or that of NADP is inhibited by aureomycin. Fig. 3 shows that NADP reduction is sensitive to aureomycin. In the light, chloroplasts shocked osmotically in 10 mM $MgCl_2$ support significant rates of electron flow to NADP even if no external ferredoxin is added. This reaction was inhibited by aureomycin.

There was the question whether electron transfer from photosystem I to ferredoxin or from reduced

ferredoxin to NADP was inhibited. Fig. 4 shows simultaneous recordings of nitrite-dependent oxygen evolution by intact chloroplasts, of chlorophyll fluorescence and of fluorescence emitted by 5 μM 9-aminoacridine in the chloroplast suspension before and after addition of aureomycin. Nitrite is known to be reduced by reduced ferredoxin. Oxygen evolution shows stimulation, not inhibition or nitrite reduction by aureomycin. This is consistent with the data obtained for methylviologen reduction (Fig. 2) and suggests some uncoupling [4]. When the nitrite reduction data are compared with the NADP reduction data, it becomes obvious that aureomycin does not inhibit ferredoxin reduction, but rather the transfer of electrons from reduced ferredoxin to NADP. It appears to interact with ferredoxin-NADP reductase.

The kinetics of chlorophyll and 9-aminocridine fluorescence further suggest some decrease of the transthylakoid proton gradient in the presence of aureomycin which might explain stimulation of electron flow to nitrite. Light dependent quenching of 9-aminoacridine fluorescence is caused by accumulation of 9-aminoacridine in the intrathylakoid space [5, 20]. It indicates formation of a transthylakoid proton gradient which is believed to be part of the driving force for ATP synthesis [21]. Decreased

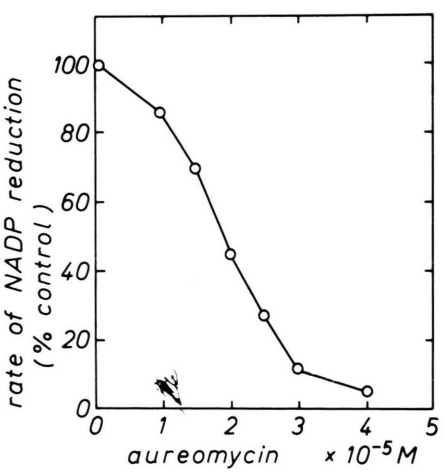

Fig. 3. Effect of aureomycin on light-dependent NADP reduction by osmotically ruptured chloroplasts. The assay medium contained 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM NADP and 25 mM HEPES buffer, pH 7.6. The control rate (100%) was 11 μmol NADPH formation/mg chlorophyll per hour. In other experiments, control rates as high as 50 μmol NADP reduction/mg chlorophyll per hour were observed.

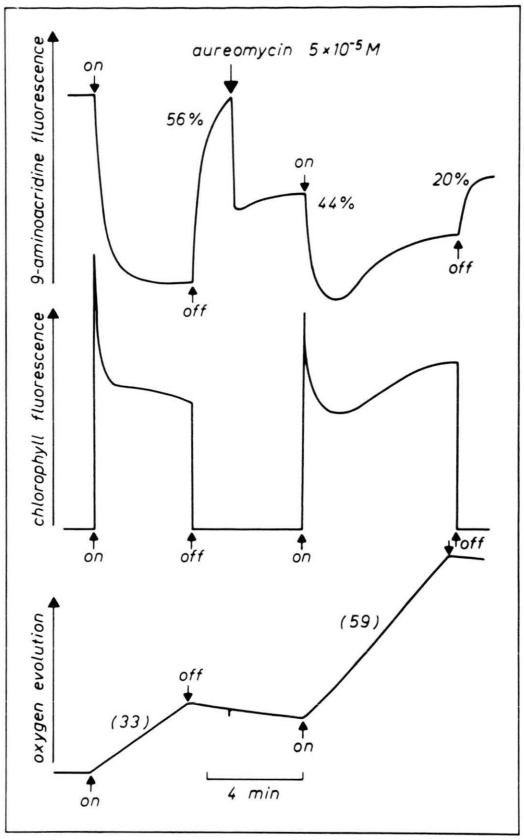

Fig. 4. Effect of aureomycin on light-dependent nitrite reduction by intact chloroplasts. Simultaneous recording of nitrite-dependent oxygen evolution, chlorophyll fluorescence and fluorescence of 9-aminoacridine. Light-dependent quenching of fluorescence emitted from 9-aminoacridine is a measure of the magnitude of the transthylakoid proton gradient. Numbers in brackets denote oxygen evolution in μmoles/mg chlorophyll per hour, percent data the extent of quenching of 9-aminoacridine fluorescence. The intensity of red light was 120 Wm^{-2}.

9-aminoacridine fluorescence quenching in the presence of aureomycin indicates a decreased ΔpH between chloroplast stroma and intrathylakoid space. In accordance with this, energy-dependent secondary quenching of chlorophyll fluorescence [22] was relieved in the presence of aureomycin (Fig. 4). In other experiments with different preparations of intact chloroplasts, 50 μM aureomycin had no significant effect on chlorophyll or 9-aminoacridine fluorescence, and 250 μM aureomycin was needed to produce the fluorescence phenomena shown in Fig. 4.

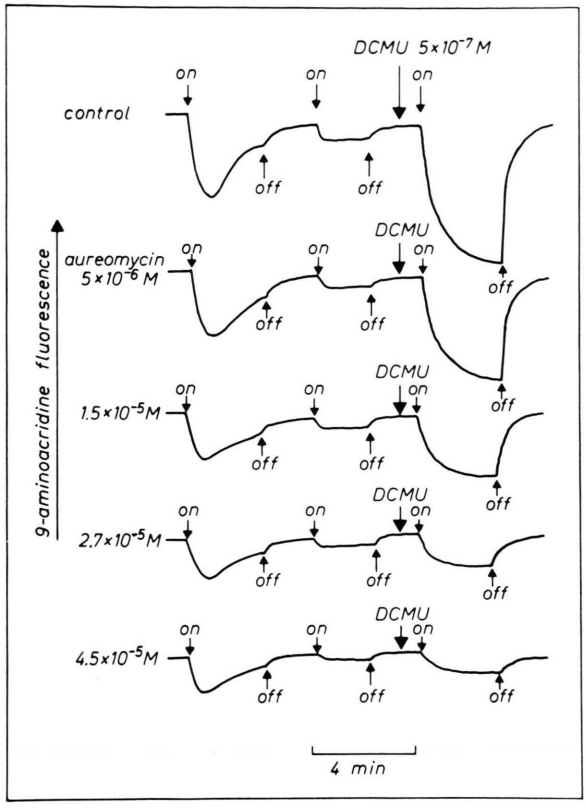

Fig. 5. Effect of DCMU and aureomycin on the fluorescence of 9-aminoacridine in a suspension of intact chloroplasts which was made anaerobic by an enzymic oxygen trap consisting of 10 mM glucose, and 50 units/ml glucose oxidase which were added to the reaction medium. The intensity of red light was 60 Wm^{-2}.

Because in intact chloroplasts cyclic electron flow has been reported to involve ferredoxin-NADP reductase [23] it was of interest to see whether cyclic electron flow is sensitive to aureomycin. Fig. 5 shows 9-aminoacridine fluorescence emitted from a suspension of intact chloroplasts. The chloroplasts were made anaerobic by the addition of an enzymic system which acts as an oxygen trap. The light-dependent formation of a proton gradient in the chloroplasts is, in the absence of electron acceptors, based on cyclic electron transport. Available CO_2 does not support linear electron transport in the absence of oxygen [24]. A first illumination of anaerobic chloroplasts produced modest quenching of 9-aminoacridine fluorescence quenching which decreased with time of illumination. Apparently, a significant proton gradient could not be maintained during illumination. A second illumination cycle produced only very little fluorescence quenching. However, when 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added which restricts electron flow from photosystem II to photosystem I, quenching of 9-aminoacridine increased dramatically. The insignificant quenching of 9-aminoacridine fluorescence by illumination in the absence of DCMU is explained by over-reduction of the electron transport chain by photosystem II which causes inhibition of cyclic electron transport [25, 26]. Inhibition was relieved by a concentration of DCMU which decreased electron pressure sufficiently to relieve over-reduction. This permitted formation of a significant proton gradient. However, in the presence of increasing concentrations of aureomycin, formation of the proton gradient in the absence of oxygen was increasingly inhibited. This may be explained by inhibition of cyclic electron transport through inhibition of ferredoxin-NADP reductase. Alternatively, aureomycin may dissipate the proton gradient more directly as indicated by decreased proton gradients in the presence of nitrite (Fig. 4) and by the stimulation of electron flow to nitrite or methylviologen (Fig. 2) which does not involve ferredoxin-NADP reductase.

However, a decreased ΔpH was not always indicated by 9-aminoacridine fluorescence in the presence of aureomycin. Fig. 6 shows a simultaneous recording of CO_2-dependent oxygen evolution by intact chloroplasts and of 9-aminoacridine fluorescence. When the fluorescence decrease brought about by the addition of aureomycin to the sample is taken into account, the magnitude of ΔpH as measured by the fluorescence method was not affected by aureomycin which completely inhibited photosynthesis. Apparently, electron transport to oxygen and residual cyclic electron transport were still sufficient to maintain a large transthylakoid proton gradient. Also, the light-induced membrane potential which is indicated by fast changes in 518 nm absorbance was not much affected by a concentration of aureomycin (50 μM) which was sufficient for CO_2 reduction. 200 or 250 μM aureomycin were required for drastic inhibition of the fast 518 nm signal (data not shown).

It is thus not clear whether inhibition of CO_2 reduction by aureomycin is caused by uncoupling or by inhibition of ferredoxin-NADP reductase. For broken chloroplasts, inhibition of photophospho-

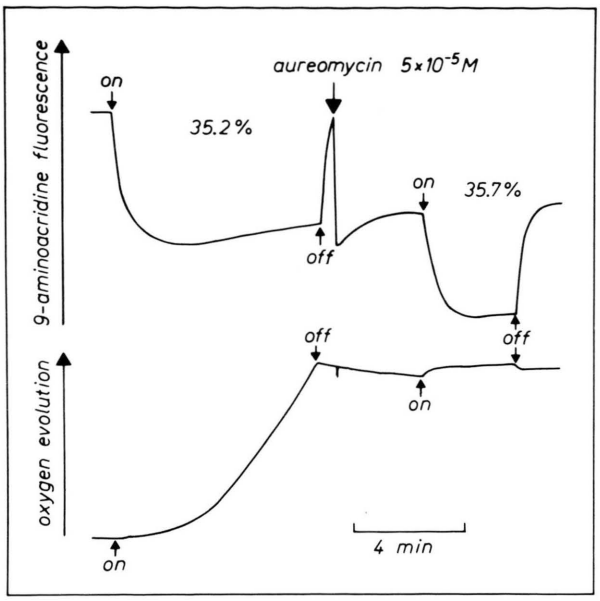

Fig. 6. Effect of aureomycin on photosynthesis of intact chloroplasts. Simultaneous recording of CO_2-dependent oxygen evolution and of 9-aminoacridine fluorescence. Addition of aureomycin caused some decrease in fluorescence emission, but the percentage of fluorescence quenched by illumination was similar before and after addition of aureomycin although CO_2-reduction was inhibited by aureomycin (see also Fig. 1). The rate of steady state O_2 evolution in the absence of aureomycin was 80 μmol/mg chlorophyll per hour, and light was rate-limiting.

rylation could be demonstrated to require more aureomycin than inhibition of CO_2 reduction (compare Fig. 7 with Fig. 1). In a number of experiments with broken chloroplasts, photophosphorylation was even less sensitive to aureomycin than shown in the experiment of Fig. 7. When ferredoxin-NADP reductase was assayed both in broken chloroplasts and as the isolated and purified enzyme by measuring the oxidation of NADPH by ferricyanide [19], enzyme inhibition was observed at aureomycin concentrations considerably in excess to those required to inhibit CO_2 reduction by intact chloroplasts. 50% inhibition of the isolated enzyme required about 300 μM aureomycin.

Thus, experiments with broken chloroplasts give no clear indication of the site of aureomycin action on photosynthesis of intact chloroplasts. However, Fig. 8 suggests that in intact chloroplasts ATP synthesis may be more sensitive to aureomycin than in broken chloroplasts. A chloroplast suspension containing phosphoglycerate as substrate was illuminated in the absence and in the presence of sufficient aureomycin to cause considerable inhibition of phosphoglycerate reduction. On illumination, NADP levels decreased and ATP levels increased both in the control and in samples containing aureomycin. Reduction of the small pool of NADP after 10 sec illumination in the presence of aureomycin corresponds to a rate of electron flow to NADP as low as about 4 μmoles reduction per mg chlorophyll per hour. It is therefore not in contradiction to inhibition of NADP reduction by aureomycin (Fig. 3). NADP remained reduced in the light, but ATP levels decreased in the sample containing aureomycin after the initial increase indicating interference of aureomycin with ATP synthesis.

The peculiar insensitivity of the proton gradient to aureomycin observed in the presence of CO_2 (Fig. 6) made it necessary to investigate also whether aureomycin possesses properties of an energy transfer inhibitor, although this did not appear likely in view of the observed stimulation of electron flow to nitrite or methylviologen. Interaction of aureomycin with the coupling factor CF_1 was reported by Huang *et al.* [11]. The latent ATPase activity of this enzyme complex was activated by illuminating broken chloroplasts in the

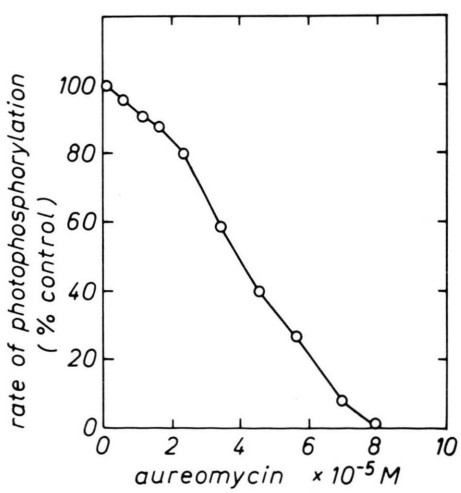

Fig. 7. Effect of aureomycin on photophosphorylation of broken chloroplasts with methylviologen as electron acceptor. The assay medium contained 5 mM $MgCl_2$, 10 mM NaCl, 2 mM K_2HPO_4 and 2 mM ADP. The reaction was started at pH 7.8 and the intensity of red light was about 400 Wm^{-2}. The control rate (100%) was 174 μmol ATP formation/mg chlorophyll per hour.

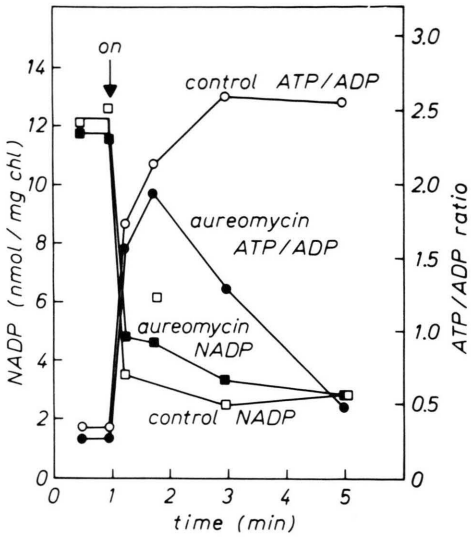

Fig. 8. Light-dependent changes in the ratios of ATP to ADP and in NADP levels in intact chloroplasts as a function of illumination time with and without 45 μM aureomycin present. The chloroplast suspension contained 1 mM 3-phosphoglycerate. The intensity of red light was 400 Wm^{-2}.

presence of phenazine methosulfate and dithiothreitol [27]. ATP added after darkening was hydrolyzed as shown by hydroxyl ion uptake according to

$$ATP^{4-} + OH^- \rightarrow ADP^{3-} + HPO_4^{2-} .$$

Aureomycin had little effect on this reaction which was inhibited by the energy transfer inhibitor Dio-9.

Conclusions

The results of this work show that aureomycin does not only bind to ribosomes but also interacts with biomembranes. In chloroplasts, the antibiotic inhibits electron flow from ferredoxin to NADP. This reaction is more sensitive to aureomycin than the reduction of ferricyanide by the isolated ferredoxin-NADP reductase. This suggests that aureomycin prevents the reduction of enzyme-bound flavine by reduced ferredoxin rather electron transfer from flavine to NADP. Aureomycin also inhibits ATP synthesis and collapses the light-induced membrane potential and the transthylakoid proton gradient, when present at concentrations above 100 μM. The latter effects cannot be explained by inhibition of electron flow which is actually stimulated when nitrite or methylviologen are electron acceptors. When CO_2 reduction is inhibited, the large proton gradient maintained in the presence of about 50 μM aureomycin (Fig. 6) can only be supported by electron flow. Electron transport to oxygen is known to be slow [28, 29], and cyclic electron transport should be subject to the same electron transport inhibition as reduction of NADP by ferredoxin-NADP reductase [23].

In this work, we have been more interested in the inhibition of photosynthesis by aureomycin than in its stimulation as shown by Huang *et al.* [10]. The observation that aureomycin possesses uncoupling properties may shed some light on reported stimulatory effects. It has been known for a long time that uncoupling can, at very low concentrations of uncoupler, stimulate both electron transport and phosphorylation. The nature of this effect is obscure [8].

Acknowledgments

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