

# Antimycin A Stimulation of Rate-limiting Steps of Photosynthesis in Isolated Spinach Chloroplasts<sup>1</sup>

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## ABSTRACT

Changes in levels of metabolites in isolated spinach (*Spinacia oleracea*) chloroplasts seen upon addition of antimycin A suggest that the activities of enzymes mediating several regulated reactions are affected. Apparently, the presence of added antimycin A does not increase the level of CO<sub>2</sub> in the chloroplasts, nor does it stimulate CO<sub>2</sub> fixation by increasing the level of the carboxylation substrate, ribulose-1,5-diphosphate. Rather, it appears that antimycin A increases CO<sub>2</sub> fixation rate by indirectly stimulating the enzyme, ribulose-1,5-diphosphate carboxylase (E.C. 4.1.1.39), which mediates the carboxylation of ribulose-1,5-diphosphate to give 3-phosphoglycerate. Another rate-limiting enzyme of the reductive pentose phosphate cycle, hexose diphosphatase (E.C. 3.1.3.11), seems also to be stimulated. The synthesis of polysaccharides (mostly starch) seems also to be stimulated. These results are interpreted as indicating that antimycin A addition enhances the general activation of those enzymes which already are activated during photosynthesis but are inactive in the dark. The ratio of adenosine triphosphate-adenosine diphosphate under conditions of photosynthesis was only moderately decreased in the presence of antimycin A, perhaps accounting in part for an observed increase in accumulation of 3-phosphoglycerate as compared with dihydroxyacetone phosphate. No significant effect on movement of metabolites from the chloroplast to the medium was seen.

Antimycin A has been reported to stimulate the rate of photosynthesis of isolated spinach chloroplasts, as measured by the rate of bicarbonate uptake (11, 12, 15, 26). Identification of the site of action of this stimulation and determination of the mechanism of stimulation could aid in the general understanding of the control of the photosynthetic metabolism.

The action of antimycin A as an inhibitor and uncoupler of photoelectron transport and phosphorylation in chloroplast fragments has been previously reported (2, 3, 14, 16, 17, 28, 29). Several observations have led to proposals that antimycin A stimulates the rate of CO<sub>2</sub> fixation in intact chloroplasts by stimulating the transport or activation of CO<sub>2</sub> or bicarbonate for the carboxylation step (12, 26).

The effects of antimycin A addition on the levels of labeled metabolites formed during photosynthesis by isolated, whole spinach chloroplasts are examined in this report. Also, the effects of antimycin A on the transport of metabolites from the chloroplasts were followed to determine if any changes in transport could be related to the stimulatory effects of antimycin A.

## MATERIALS AND METHODS

Chloroplasts were isolated from spinach (*Spinacia oleracea*) leaves, and the rate of <sup>14</sup>CO<sub>2</sub> fixation was determined as previously described, with the exception that unless noted, isoascorbate was omitted from all solutions (18). Analysis of the <sup>14</sup>C and <sup>32</sup>P-labeled products of photosynthesis by two-dimensional chromatography and radioautography was carried out as described earlier (23). Estimation of the distribution of metabolites between the chloroplasts and the surrounding medium was also carried out as previously described (8). The <sup>14</sup>C radioactivity found at the origin was taken as a measure of the synthesis of <sup>14</sup>C-labeled starch and other polysaccharides. With whole cells, following photosynthesis with <sup>14</sup>CO<sub>2</sub>, acid hydrolysis of this insoluble material at the origin gives glucose, amino acid, and nucleic acid components (20, 21). Spinach chloroplasts, isolated and allowed to photosynthesize with <sup>14</sup>CO<sub>2</sub> as described previously (12), produce essentially only polysaccharides as insoluble end products, since such chloroplasts form only negligible amounts of amino acids.

Chlorophyll was determined by the method of Arnon (1).

Antimycin A was obtained from Sigma Chemical Co. and dissolved in absolute ethanol. The total concentration of ethanol was kept at 1% or less, and, unless indicated, all controls contained 1% ethanol.

## RESULTS

In the earlier reports of the antimycin A stimulation of CO<sub>2</sub> fixation with chloroplasts, the control rates were generally low—10 to 35 μmoles CO<sub>2</sub> fixed/mg chl·hr (11, 12, 26). An early observation in the present study was that chloroplasts with CO<sub>2</sub> fixation rates of 130 μmoles/mg chl·hr were stimulated by 0.5 μM antimycin A to CO<sub>2</sub> fixation rates of 180 μmoles/mg chl·hr. While the control rates and the magnitude of the antimycin A stimulation varied with the spinach used, the presence of antimycin A (0.5–5.0 μM) in the incubation mixture from the start of the experiment dependably gave large stimulations over the control rate. A typical result is given in Figure 1. In the case where antimycin A was added at the beginning of the experiment the CO<sub>2</sub> fixation rate (118 μmoles/mg chl·hr) during the first 5 min was stimulated 79% over the control rate. When antimycin A was added after 5-

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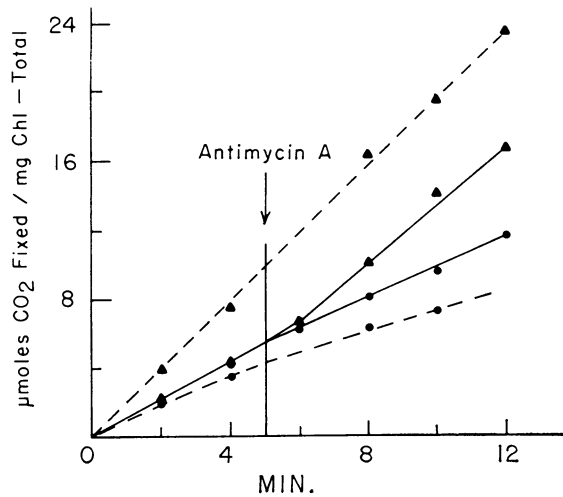


FIG. 1. Rates of  $\text{CO}_2$  fixation by isolated spinach chloroplasts with added antimycin A. ● (broken line): Control with  $10 \mu\text{l}$  of ethanol added (rate =  $66 \mu\text{moles CO}_2/\text{mg chl}\cdot\text{hr}$  during first 5 min); ● (solid line): control with  $10 \mu\text{l}$  of ethanol added at 5 min (rate =  $53 \mu\text{moles CO}_2/\text{mg chl}\cdot\text{hr}$  during 7- to 12-min interval); ▲ (broken line): antimycin A added at the beginning of experiment (rate =  $118 \mu\text{moles CO}_2/\text{mg chl}\cdot\text{hr}$  during first 5 min); ▲ (solid line): antimycin added at 5 min (rate =  $106 \mu\text{moles CO}_2/\text{mg chl}\cdot\text{hr}$  during 7- to 12-min interval).

min photosynthesis, the subsequent rate ( $106 \mu\text{moles}/\text{mg chl}\cdot\text{hr}$ ) was 100% higher than the control rate ( $53 \mu\text{moles}/\text{mg chl}\cdot\text{hr}$ ).

The experiment which gave the data shown in Figure 1 was performed with no preincubation and an argon gas phase. Addition of antimycin A to chloroplasts after the usual light, air, preincubation and 2 to 5 min photosynthesis in the presence of bicarbonate sometimes gave little or no stimulation. Exclusion of air seems to be required to obtain the stimulation reproducibly. Moreover, the inclusion of 10 mM isoascorbate, usually a standard component of the incubation mixture for chloroplasts (18), also prevented the stimulatory response to antimycin A, even if the antibiotic were initially present. Unless noted, therefore, isoascorbate was routinely omitted from the reaction mixture, and an argon atmosphere was provided.

The effects of the addition during photosynthesis of antimycin A on the incorporation of  $^{14}\text{C}$  into several intermediates of the Calvin cycle are shown in Figures 2 to 8. The increase in 3-PGA and RuDP, and the drop in SDP and in FDP, occurred consistently upon the addition of antimycin A, but the increase in starch and decrease in F6P occurred only if the addition caused an increase in the rate of fixation.<sup>4</sup>

If antimycin A were simply increasing the effective concentration of the  $\text{CO}_2$  substrate of the RuDP carboxylase, the level of RuDP would drop upon stimulation. This did not occur (Fig. 4). In order to test whether or not a higher level of  $\text{CO}_2$  inside the chloroplasts could produce effects similar to those caused by antimycin A addition, the incorporation of  $^{14}\text{C}$  into the Calvin cycle intermediates accompanying a switch from low to high bicarbonate concentrations were studied. The changes in RuDP and SDP levels resulting from such in-

creased bicarbonate (Figs. 9 and 10) are completely different from the changes due to antimycin A addition (Figs. 4 and 5). The increase in SDP with increased bicarbonate concentration was typical of the changes in all intermediates examined (starch, PGA, DHAP, FDP, F6P, etc.) except for RuDP, which clearly, and as predicted, dropped upon increasing the bicarbonate concentration.

As previously reported (12, 26), antimycin A stimulated the rate of  $\text{CO}_2$  fixation at both limiting and saturating bicarbonate concentrations. The metabolic consequences of added antimycin A were also similar at both bicarbonate concentrations, as shown by the levels of SDP and RuDP in Figures 11 and 12.

Since it had been demonstrated that regulatory factors ( $\text{PP}_i$ ,  $\text{Mg}^{2+}$ , and a factor associated with spinach alkaline FDPase) can have marked effects on the transport or release of metabolites from the chloroplasts into the surrounding medium (6), it was of interest to determine if antimycin A might be exerting its stimulatory effects by changing the pattern of transport of metabolites from the chloroplasts. An examination of the distribution between chloroplasts and the surrounding medium of the Calvin cycle intermediates labeled during photosynthesis showed no significant changes in movement of metabolites into the medium in response to antimycin A.

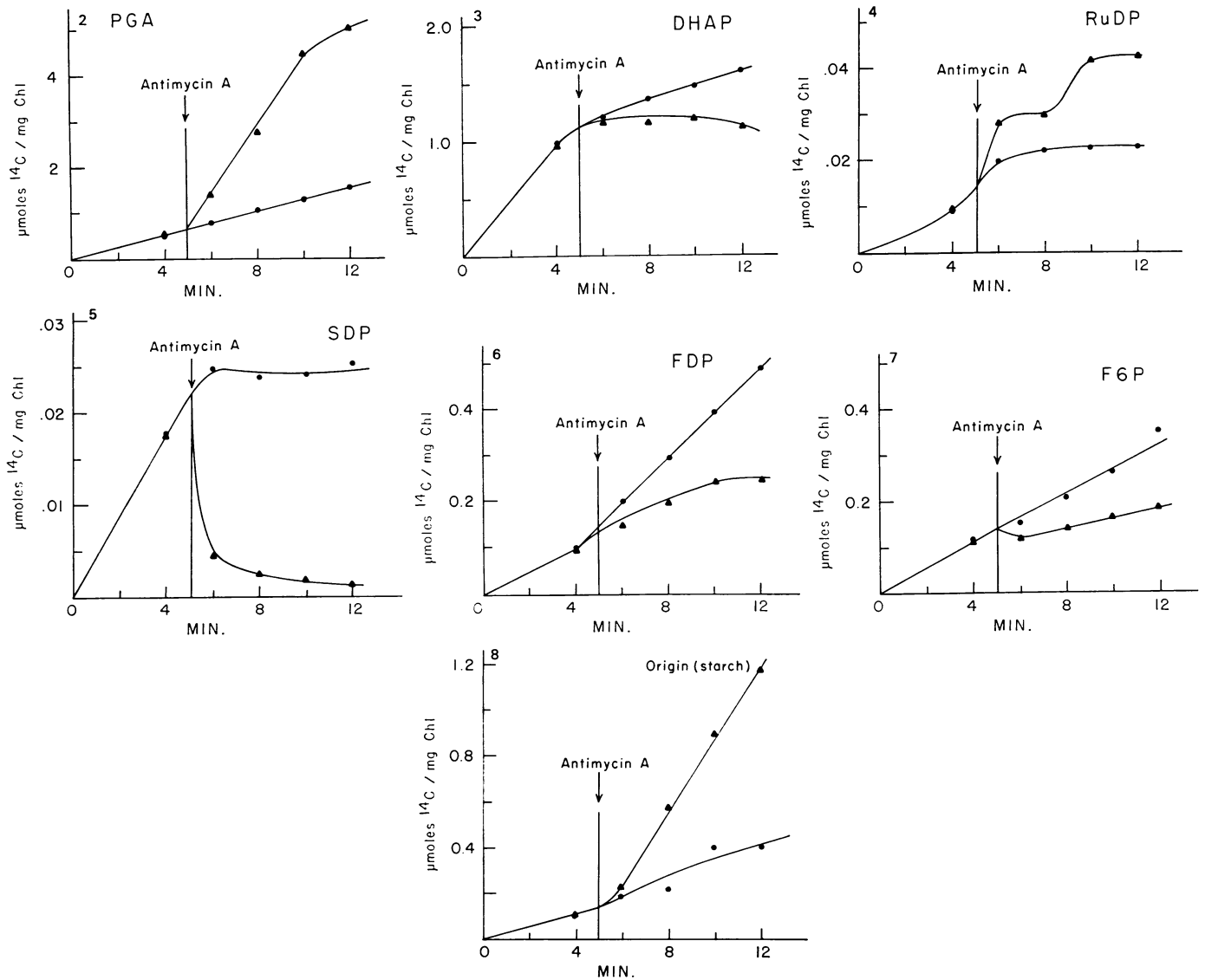
Because antimycin A has frequently been demonstrated to uncouple both cyclic and noncyclic photophosphorylation in chloroplast fragments (14), we examined the effect of antimycin A on the levels of ATP in intact chloroplasts before and during photosynthesis by following the incorporation of  $^{32}\text{P}_i$  into ATP both in the presence and absence of bicarbonate. The data presented in Figure 13 were obtained by incubating chloroplasts in the presence and absence of  $0.5 \mu\text{M}$  antimycin A, first during an air, light preincubation, and then after the addition of bicarbonate. There was no significant change in the accumulation of  $^{32}\text{P}_i$  into ATP in the absence of bicarbonate. Upon addition of bicarbonate, a slightly greater decrease in the labeled pool of ATP was seen in the chloroplasts incubated with antimycin A. Since the rate of  $\text{CO}_2$  fixation was increased in this instance by antimycin A from 74 to  $141 \mu\text{moles fixed}/\text{mg chl}\cdot\text{hr}$ , the actual rate of synthesis of ATP must be correspondingly greater in the chloroplasts incubated with antimycin A than in the control chloroplasts. Thus the decreased level of ATP might be due only to the increased demand for ATP compared to its rate of synthesis. This may also account for the drop in labeled ATP observed upon the addition of antimycin A to photosynthesizing chloroplasts (Fig. 14).

It was also found that the stimulation of  $\text{CO}_2$  fixation by antimycin A is somewhat sensitive to the concentration of added  $\text{Mg}^{2+}$  (Fig. 15). Stimulation of  $\text{CO}_2$  fixation by  $0.5 \mu\text{M}$  antimycin A decreased appreciably with increasing  $\text{Mg}^{2+}$  concentration above about  $0.3 \text{ mM}$   $\text{Mg}^{2+}$ . The stimulation by  $5 \mu\text{M}$  antimycin A was relatively insensitive to added  $\text{Mg}^{2+}$ . Unlike the inhibitory fraction obtained from spinach juice (6), antimycin A is not synergistically inhibitory with  $\text{Mg}^{2+}$ .

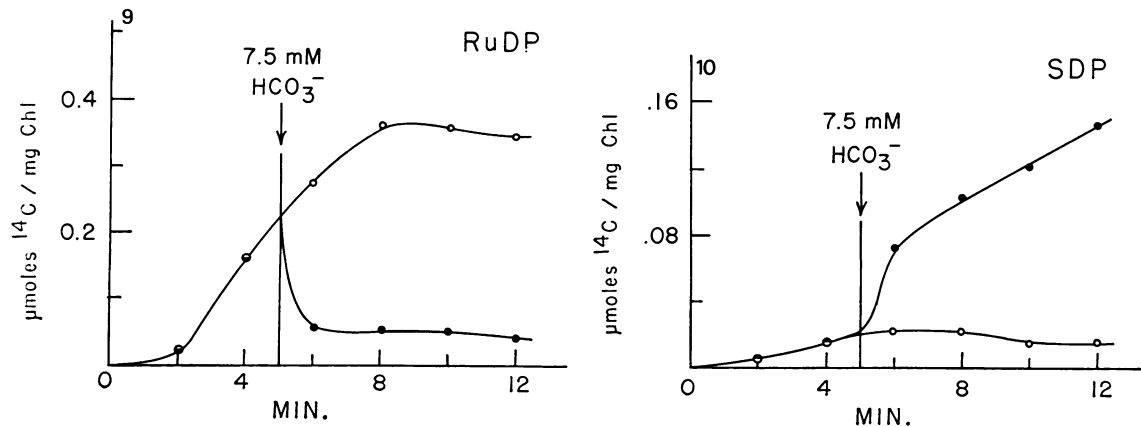
## DISCUSSION

That antimycin A might increase the chloroplasts' efficiency for utilization of bicarbonate had been suggested primarily by the following observations: (a) the increase in both the absolute and relative amount of synthesis of PGA in the presence of antimycin A (11, 26); (b) the decrease in the  $K_m(\text{HCO}_3^-)$  of the chloroplasts in the presence of antimycin A (12, 26); (c) the apparent insensitivity of the RuDP carboxylase to antimycin A when assayed in osmotically lysed chloroplasts (26); (d) the fact that the  $K_m(\text{HCO}_3^-)$  of the carboxylase is

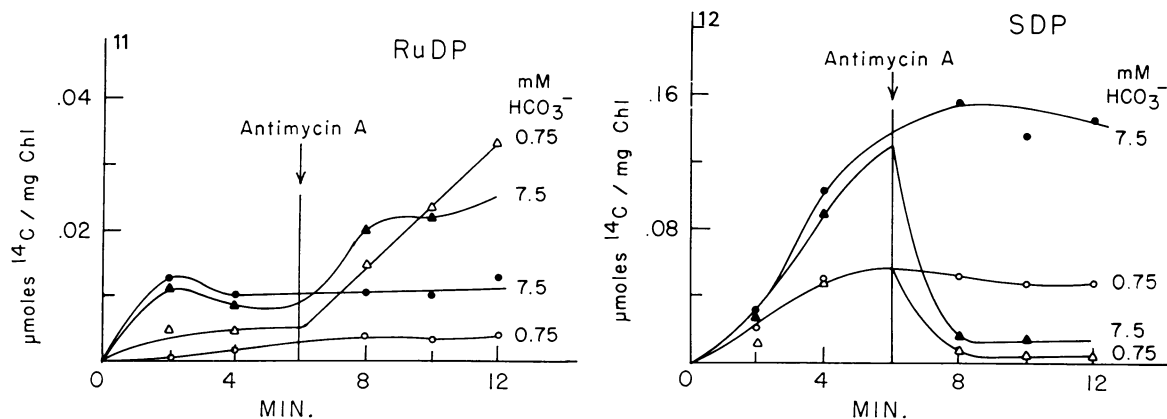
<sup>4</sup> Abbreviations: DHAP: dihydroxyacetone phosphate; FDP: fructose-1,6-diphosphate; FDPase: fructose-1,6-diphosphatase (E.C. 3.1.3.11); F6P: fructose-6-phosphate; PGA: 3-phosphoglycerate; RuDP: ribulose-1,5-diphosphate; RuDP carboxylase: ribulose diphosphate carboxylase (E.C. 4.1.1.39); SDP: sedoheptulose-1,7-diphosphate.



Figs. 2 thru 8. Effects on <sup>14</sup>C-labeled metabolites of addition of antimycin A to photosynthesizing isolated spinach chloroplasts. Antimycin A was added after 5 min of photosynthesis, as described in text. ▲: With added antimycin A; ●: control. 2: 3-Phosphoglyceric acid; 3: dihydroxyacetone phosphate; 4: ribulose-1,5-diphosphate; 5: sedoheptulose-1,7-diphosphate; 6: fructose-1,6-diphosphate; 7: fructose-6-phosphate; 8: origin (starch).



Figs. 9 and 10. Effects of increasing H<sup>14</sup>CO<sub>3</sub><sup>-</sup> concentrations on levels of labeled metabolites in photosynthesizing isolated spinach chloroplasts. After 5-min photosynthesis with 0.75 mM H<sup>14</sup>CO<sub>3</sub><sup>-</sup>, the concentration was increased to 7.5 mM H<sup>14</sup>CO<sub>3</sub><sup>-</sup>. ○: Both flasks before increase in HCO<sub>3</sub><sup>-</sup>; ●: HCO<sub>3</sub><sup>-</sup> concentration increased to 7.5 mM at 5 min. 9: Ribulose-1,5-diphosphate; 10: sedoheptulose-1,7-diphosphate.



FIGS. 11 and 12. Effects of high and low concentrations of  $\text{H}^{14}\text{CO}_3^-$  on levels of labeled metabolites in photosynthesizing isolated spinach chloroplasts in the presence and absence of added antimycin A.  $\Delta$ ,  $\blacktriangle$ : With antimycin A added;  $\circ$ ,  $\bullet$ : controls. 11: Ribulose-1,5-diphosphate; 12: sedoheptulose-1,7-diphosphate.

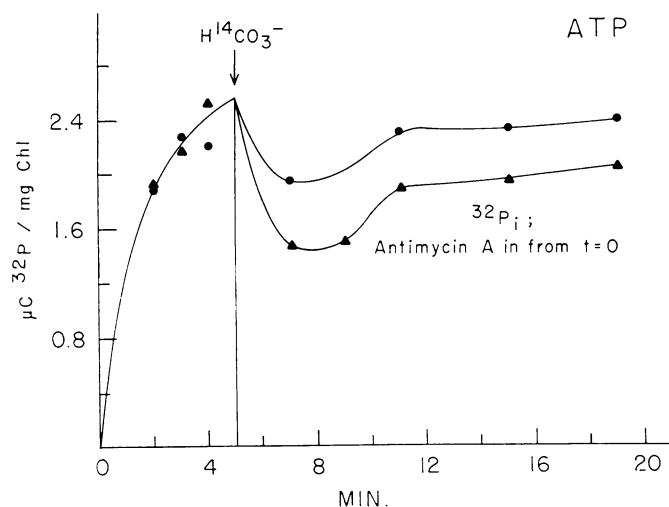


FIG. 13. Levels of  $^{32}\text{P}$ -labeled ATP in isolated spinach chloroplasts with added antimycin A. Isolated spinach chloroplasts were illuminated without added  $\text{H}^{14}\text{CO}_3^-$  in the presence of  $^{32}\text{P}$ -labeled inorganic phosphate. After 5 min,  $\text{H}^{14}\text{CO}_3^-$  was added to the control flask and to the flask containing antimycin A.  $\blacktriangle$ : Antimycin A added;  $\bullet$ : control.

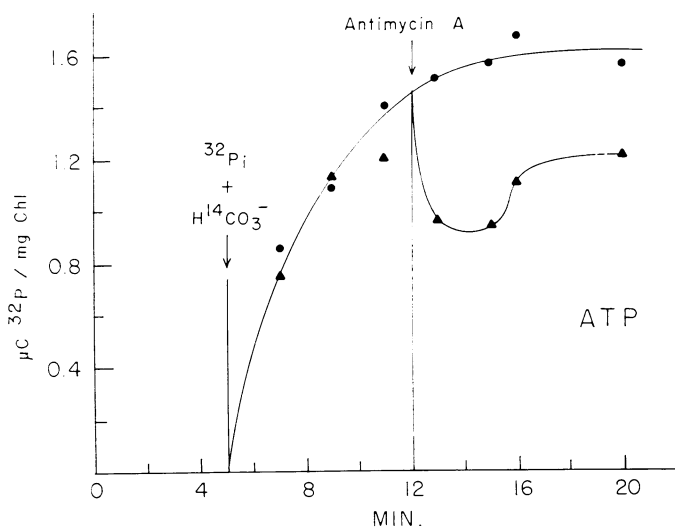


FIG. 14. Effect of added antimycin A on the level of  $^{32}\text{P}$ -labeled ATP in photosynthesizing isolated spinach chloroplast.  $\blacktriangle$ : Antimycin A added;  $\bullet$ : control.

higher than the  $K_m(\text{HCO}_3^-)$  of the chloroplasts (26). These facts, plus the demonstration by Cooper *et al.* (13) that  $\text{CO}_2$ , not  $\text{HCO}_3^-$ , was the substrate for the carboxylase led to the hypothesis that such a transport or activation step for bicarbonate existed in the intact chloroplasts and was the step primarily stimulated by antimycin A (26).

The present study shows the level of RuDP does not drop, but in fact rises upon the addition of antimycin A to photosynthesizing chloroplasts (Fig. 4), whereas RuDP level does drop upon addition of more bicarbonate (Fig. 9). Thus, it is clear that the mechanism of stimulation of  $\text{CO}_2$  fixation rate by antimycin A is not by increasing  $\text{CO}_2$  level inside the chloroplast. Even though the level of  $\text{CO}_2$  inside the chloroplast may not exactly reflect the bicarbonate concentration in the medium, the dramatic drop in RuDP level accompanying the increase in  $\text{HCO}_3^-$  in the medium shows that  $\text{CO}_2$  level inside has increased and that such an increase immediately affects RuDP level.

It might be argued that the somewhat increased level of RuDP seen in the presence of antimycin A is responsible for the increased rate of  $\text{CO}_2$  fixation. However, many studies in this laboratory (*e.g.*, ref. 6) have provided evidence that  $\text{CO}_2$  fixation rate is quite insensitive to RuDP levels in the range usually observed. Often a higher level of RuDP in photosyn-

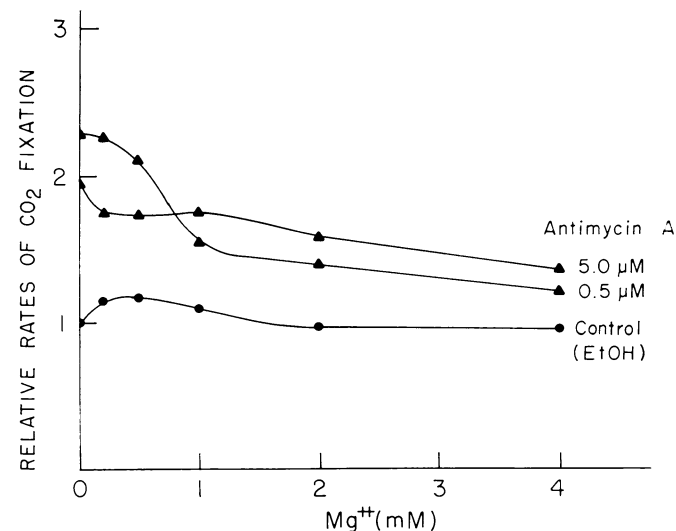


FIG. 15. Effect of  $\text{Mg}^{2+}$  ion concentrations on antimycin A stimulated photosynthesis by isolated spinach chloroplasts.

thesizing chloroplasts is indicative of slower CO<sub>2</sub> fixation rate. The level of RuDP under limiting bicarbonate is clearly sufficient to support a higher rate of carboxylation (Fig. 9), as evidenced by the increase in the rate of CO<sub>2</sub> fixation from 19 to 65  $\mu$ moles <sup>14</sup>CO<sub>2</sub> fixed/mg chl·hr upon the addition of increased bicarbonate. Since antimycin A will increase the rate of carboxylation even under limiting bicarbonate, it would appear that the level of RuDP is not a rate-limiting factor responsible for the response to antimycin A. The carboxylation reaction has been identified as a primary site of metabolic regulation in the reductive pentose phosphate cycle (4, 19, 20, 23, 24), and studies of the properties of the isolated enzyme, RuDP carboxylase, show regulatory properties (10, 27, 30). The steady-state free energy change for the *in vivo* carboxylation reaction in *Chlorella pyrenoidosa* has been calculated as about -10 kcal (9). Therefore it is not surprising that the reaction rate is insensitive to RuDP concentration.

If antimycin A stimulation of fixation occurs neither by increased CO<sub>2</sub> level or RuDP level in the chloroplasts, stimulation of the enzymic activity of RuDP carboxylase remains the only other possible explanation for the increased rate of fixation of CO<sub>2</sub>.

The large drop in levels of SDP upon antimycin A addition (Figs. 5 and 12) strongly suggests an activation of the enzyme, hexose diphosphatase, which catalyzes the hydrolysis of SDP and of FDP to the respective monophosphates. This is also suggested by the drop in level of FDP, even though the relative effect appears smaller. The reason that the drop in FDP is smaller is that most of the FDP present is in the medium outside the chloroplasts (6) where FDP can be formed from triose phosphates in the medium. Because of this, the total pool of FDP is much larger than that of SDP. The actual difference in  $\mu$ moles of <sup>14</sup>C incorporated, 5 min after addition of antimycin A, is greater for FDP than for SDP.

We conclude, therefore, that the reactions mediated by hexose diphosphatase and by RuDP carboxylase can be stimulated by the presence of antimycin A. The stimulation of RuDP carboxylase, leading to an increased rate of CO<sub>2</sub> fixation, appears only under certain conditions. These reactions are the rate-limiting steps in CO<sub>2</sub> fixation *in vivo* (4, 19, 20, 22, 23) and are the steps inactivated in the dark and metabolically controlled in the light.

It has been proposed that pH in the chloroplast stroma region, together with Mg<sup>2+</sup> ion levels, may affect the activities of these enzymes (4, 7). Other general regulatory mechanisms associated with photoelectron transport in the chloroplast thylakoids are also possible. Such regulatory mechanisms might be influenced by the action of antimycin A on properties of the photochemical apparatus, such as photoinduced ion flux in the chloroplast membranes. Thus, antimycin A could be stimulating some light-induced process which activates the regulated enzymes of the reductive pentose phosphate cycle. It could be acting in a direction opposite from that of octanoate which reversibly abolished this light-activating process (22).

The stimulation of starch synthesis by antimycin A also suggests enhancement of a general light-activated stimulation. The properties of the enzyme, ADP-glucose pyrophosphorylase strongly suggested that the reaction of glucose-1-P with ATP to give ADP-glucose may be a site of metabolic control (24, 25). Kinetic studies of *in vivo* metabolism in *Chlorella pyrenoidosa* gave results that suggest that the activity of ADP glucose pyrophosphorylase, like that of hexose diphosphatase and RuDP carboxylase, must greatly decrease within a minute after the light is off (5, 21). The present study suggests that antimycin A stimulates all three enzymes.

The stimulation of starch synthesis by this mechanism

could account for the drop in level of F6P even when hexose diphosphatase has been stimulated.

The results in this study do not provide any convincing evidence of an effect of antimycin A on the rate of ATP synthesis. The total incorporation of <sup>32</sup>P into organic phosphates is actually increased with antimycin A (26), but the increase is less than the increase in CO<sub>2</sub> fixation, since some <sup>14</sup>C is incorporated into starch and other nonphosphorylated products. The level of <sup>32</sup>P-labeled ATP during CO<sub>2</sub> fixation is about 25% lower with antimycin A than without. This could be due only to the increased requirement for ATP occasioned by the stimulation of CO<sub>2</sub> fixation if the stimulation of ATP synthesis is less than the stimulatory effects on the regulated enzymes.

The lower level of ATP in the presence of antimycin A, whatever its reason, could be partly responsible for the accumulation of PGA. The two-step phosphorylation and reduction of PGA to triose phosphate is fairly reversible *in vivo* (9) ( $\Delta G^\circ = -1.6$  kcal), so that a 25% decrease in ATP level, and a corresponding increase in ADP, could shift the steady-state ratios of forward and back reactions. Since some of the PGA comes out of the chloroplasts into the medium (8), a small increased imbalance between rate of PGA formation and utilization could lead to a build up of PGA in the chloroplasts and a significantly increased rate of movement of PGA into the medium where it would accumulate. In the experiment which gave the data shown in Figures 1 and 2, the level of PGA 5 min after antimycin A addition was increased by 3.2  $\mu$ moles of <sup>14</sup>C relative to the control, whereas the total <sup>14</sup>CO<sub>2</sub> fixation increased by 5  $\mu$ moles in the chloroplasts with antimycin A as compared with the control. About 0.5  $\mu$ mole of the extra <sup>14</sup>C has appeared at the origin, mostly as starch (Fig. 8). The remaining extra 1.3  $\mu$ moles of <sup>14</sup>C accumulated during 5 min after antimycin A addition was distributed around a variety of weakly labeled compounds, including various oligosaccharides.

The abolition of some of the stimulation of CO<sub>2</sub> fixation by antimycin A by higher levels of Mg<sup>2+</sup> ions in the medium could be due to a variety of reasons including effects of Mg<sup>2+</sup> on movement of metabolites from the chloroplasts to the medium (6) and the known effects of Mg<sup>2+</sup> ion on the activity of RuDP carboxylase (10, 27).

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