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Relationship between the Level of Adenine Nucleotides and the Carboxylation Activity of Illuminated Isolated Spinach Chloroplasts

A STUDY WITH ANTIMYCIN A

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ABSTRACT

The changes in the levels of intact spinach (*Spinacia oleracea* L.) chloroplast adenine nucleotides during the time course of light-dependent CO_2 fixation were determined with respect to the effect of antimycin A. This study demonstrated that antimycin A lowered the rate of ATP formation during the induction period of carboxylation. While the steady state levels of ATP and the energy-charge value also decreased in the presence of antimycin, the concomitant increase of the CO_2 fixation activities insured higher ATP turnover rates. Changes in the labeling of CO_2 fixation products during the lag phase suggested a stepwise activation of the Calvin cycle, with fructose 1,6-diphosphate, and ribulose 5-phosphate kinase being activated before ribulose 1,5-diphosphate carboxylase. The possible mechanisms of the enhancement of CO_2 fixation activity by antimycin A in relation to its action on photophosphorylation during the lag phase are discussed.

Antimycin A was shown by Ellyard (18) and by Champigny and Gibbs (10) to enhance the rates of photosynthetic CO_2 uptake, O_2 evolution and inorganic phosphate esterification of intact spinach chloroplasts. At the same time, it affects the products of photosynthesis, bringing about a shift in the distribution of accumulated carbon in favor of glycerate-3-P instead of triose-P and fructose-1,6-diP.

On the other hand, antimycin A is known to be an inhibitor or uncoupler of photoelectron transport and photophosphorylation in chloroplast fragments (2, 17, 23). It was also found by Schurmann, *et al.* (38) to inhibit endogenous cyclic photophosphorylation in intact chloroplasts. Miginiac-Maslow (27) reported that in intact spinach chloroplasts the endogenous anaerobic phosphorylation is inhibited by antimycin A to the same extent as the O_2-dependent noncyclic phosphorylation.

Because the antimycin A-dependent accumulation of carbon in glycerate-3-P during the steady state could be shifted to triose-P by increasing the level of inorganic phosphate in the medium (11, 28), it was assumed that the stimulation of CO_2 fixation was in some way related to the deficiency of ATP synthesis. On the basis of this assumption, Miginiac-Maslow and Champigny (28) speculated that CO_2 assimilation might depend on a high energy precursor.

Taking into account the changes in metabolite levels, Schacter and Bassham (36) suggested that the activities of ribulose-1,5-diP carboxylase and fructose-1,6-diPase, both light-activated enzymes, might be stimulated in the presence of antimycin A, thus controlling the rate of carboxylation. From the Michaelis-Menten kinetics applied to the carboxylating chloroplasts, Schacter *et al.* (37) and Champigny and Miginiac-Maslow (12) showed that the apparent affinity of intact chloroplasts for CO_2 is increased by antimycin A, and Schacter *et al.* (37) suggested that the antimycin A function might be at the outer membrane of the chloroplast which had been assumed to contain the enzyme mechanism for setting the pace of CO_2 (20).

Thus it seemed important to establish the true nature of the effect of antimycin A on the ATP level of photosynthesizing chloroplasts. For this reason we have investigated the changes in the levels of ATP, ADP, and AMP, together with the amount of CO_2 fixed in illuminated chloroplasts. The addition of antimycin A at various concentrations permitted us to obtain the different levels of carboxylation activity from a single chloroplast preparation.

This study demonstrates that antimycin A lowers the photophosphorylation activity of chloroplasts during the induction period of carboxylation, and suggests that the stimulation of the steady state carboxylation activity would be induced during the first minute of illumination as a result of the decreased ATP/ADP ratio. This does not exclude the possibility of the participation of other, not yet demonstrated factors such as the pH of the stroma or the level of NADPH.

The changes in the labeling of some products of CO_2 fixation during the lag phase suggest that the enhancement of the fructose-1,6-diPase and of the ribulose-5-P kinase activities may take place before the increase of the carboxylation rates. During the steady state phase, the effect of antimycin is characterized by higher rates of CO_2 fixation and ATP turnover, higher PGA-triose phosphate ratios, but by a lower ATP level and a decreased energy charge value.

During the lag phase, the means by which antimycin A could induce the enhancement of the steady state carboxylation activity of intact chloroplasts are discussed on the basis of either a photosynthetic control or a possible increase of the stroma pH.

MATERIALS AND METHODS

Plant Material and Chloroplasts Isolation. Chloroplasts were isolated from spinach (*Spinacia oleracea* L.) grown on vermiculite and nutrient solution (12) in a growth chamber (12 hr light, 1500 ft-c, 23 C; 12 hr darkness, 18 C). The preparation was carried out according to the method of Cockburn *et al.* (13), with a slight modification in the homogenization medium from which isoascorbate was omitted. It contained: 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and was adjusted to pH 6.5. After isolation, the chloroplasts were suspended in a small volume of the reaction mixture (see below) from which bicarbonate was omitted. The Chl content was determined by the method of Bruinsma (7).

Conditions of Incubation. The measurements of CO_2 fixation and of adenine nucleotides were made simultaneously in two sets of vessels from the same chloroplast preparation. CO_2 fixation was carried out under air in saturating light (6000 ft-c), after a 2 min dark preincubation of chloroplasts in the reaction mixture, which contained: 0.33 M sorbitol, 50 mM HEPES at pH 7.6, 0.15 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA (disodium salt). CO_2 was supplied as $NaHCO_3$ at a concentration of 5 mM. Antimycin A in ethanol solution was added, at concentrations indicated in the figures, before the dark preincubation. The same amount of ethanol was added to every flask.

Determination of the Amount of CO_2 fixed. CO_2 fixation was studied by the ^{14}C method using $NaH^{14}CO_3$ at 4 μc/μmole. Samples (0.5 ml) of a chloroplast suspension were withdrawn periodically, as indicated in the figures, into tests tubes containing 30 μl of formic acid for fixing. Radioactivity was counted on 50 μl samples after drying, with a Nuclear Chicago gas flow counter.

Analysis of the Products of Photosynthesis. For the analysis of the ^{14}C-products, $NaH^{14}CO_3$ was at 10 μc/μmole. ^{14}C-Containing compounds were separated on Whatman No. 3MM paper by one-dimensional descending chromatography in the solvent GW 3 described by Wood (43). After autoradiography the ^{14}C content of each compound was measured by eluting the radioactivity from the paper and counting in a Packard liquid scintillation counter.

Estimation of Adenine Nucleotides. One-ml samples of chloroplast suspension were withdrawn periodically, as indicated in the figures, into centrifugation tubes containing 100 μl of 1.5 M H_2SO_4. The mixtures were centrifuged for 2 min at 16,000 rpm. The adenine nucleotide content of chloroplasts was estimated on the supernatant, after neutralization with 2 M K_2CO_3, by the luciferin-luciferase assay according to Strehler and Totter (40) as modified by Pradet (33). ATP was directly determined. ADP and AMP were assayed after enzymatic transformation into ATP (33). The maximum light emission was measured with a Farrand spectrofluorometer.

Results are given as a mean of three determinations. For two

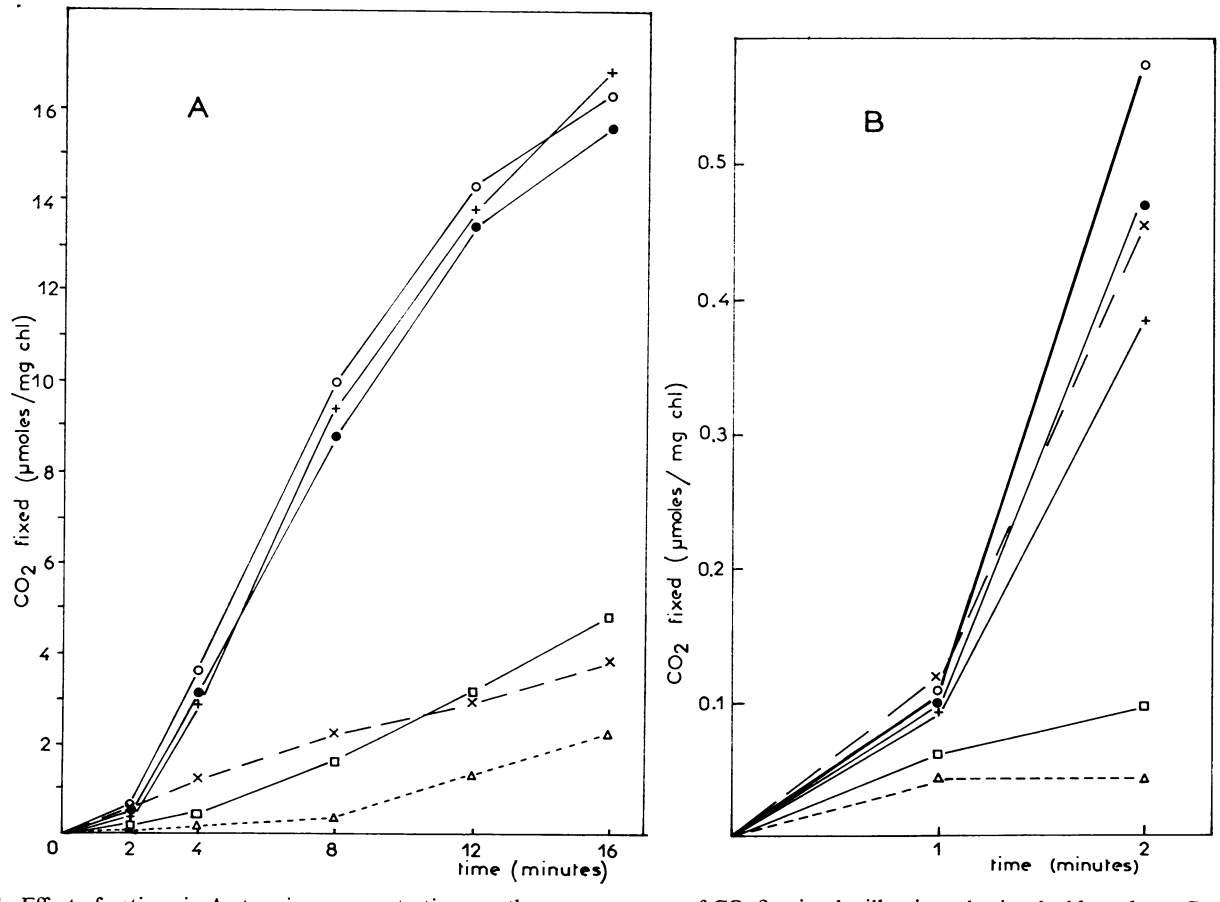

FIG. 1. Effect of antimycin A at various concentrations on the progress curve of CO_2 fixation by illuminated spinach chloroplasts. Components of the reaction mixture were as described in text under "Material and Methods." Chl concentration was 145 μg/ml and antimycin A was at concentrations indicated. B is a higher magnification of the first 2 min of A. Antimycin A treatments and rates of CO_2 fixation during 4 to 12 min intervals: control, 5.6 μmoles CO_2/mg Chl·hr (×); 1 μM antimycin, 32.2 μmoles CO_2/mgChl·hr (○); 5 μM antimycin A, 35 μmoles CO_2/mg Chl·hr (●); 10 μM antimycin A, 33.2 μmoles CO_2/mg Chl·hr (+); 50 μM antimycin A, 11.5 μmoles CO_2/mg Chl·hr (□); 100 μM antimycin A, 6.2 μmoles CO_2/mg Chl·hr (△).

different samples from the same batch of chloroplasts, ATP values agree within ± 9%, ATP + ADP values within ± 10%, ATP + ADP + AMP values within ± 18%. ADP, AMP, and energy charge, which are calculated from the preceding values, agree within ± 18%, ± 50%, ± 16%, respectively.

RESULTS AND DISCUSSION

CO_2 Fixation. If we consider the progress curve of CO_2 fixation, we can see that the response to antimycin A is not the same during the steady state (Fig. 1A) and during the lag phase (Fig. 1B). During the first minute of the induction lag, the amount of CO_2 fixed is decreased in the presence of antimycin. The extent of the decrease is dependent on the antimycin concentration. The end of the initial induction period is characterized by an increase in the rate of carboxylation, which tends to reach the maximal rate of the steady state. The time taken to reach this maximal rate depends on the depression of CO_2 fixation during the first minute of induction, the greater the depression, the longer the time of induction. If we consider that the induction period is due to a temporary deficiency of phosphorylated substrates, either sugars or ATP (38, 41), we assume that antimycin A enhances the deficiency. However, subsequently, and despite this deficiency, the rate of the steady state is increased. Except for the highest concentration of 100 μM, which completely blocks CO_2 fixation as already shown (2), antimycin A seems to induce maximal rates higher than those of the control chloroplasts.

Adenine Nucleotides and Energy Charge. In the study of the changes in the adenine nucleotide level of illuminated carboxylating chloroplasts, the induction period shorter than 2 min must be distinguished from the steady state period.

During the first five sec of illumination, ATP synthesis is decreased by antimycin (Fig. 2A). Although the difference between the control chloroplasts and the 1 μM antimycin-treated chloroplasts is not significant, decreases induced by the other three concentrations of antimycin are very significant. The decrease seems to depend on the concentration of antimycin. As a result of the lowered ATP level, the amount of CO_2 fixed assayed in the same experiment is depressed (Fig. 2B). It is interesting to note that at 1 min illumination, CO_2 fixation is exponentially related to the level of the chloroplast ATP (Fig. 3). These results support the view that during the induction period, the rate of CO_2 assimilation by chloroplasts is sustained by their level of ATP. Then, antimycin-dependent ATP deficiency may be assumed to be responsible for the lowered rate of CO_2 fixation during the induction period.

These observations are valuable only for the induction period. Indeed the relations between ATP level and CO_2 fixation activity during the steady state contrast markedly. Except for the highest concentration of antimycin, which seems to be a potent inhibitor of ATP synthesis and of CO_2 fixation, higher

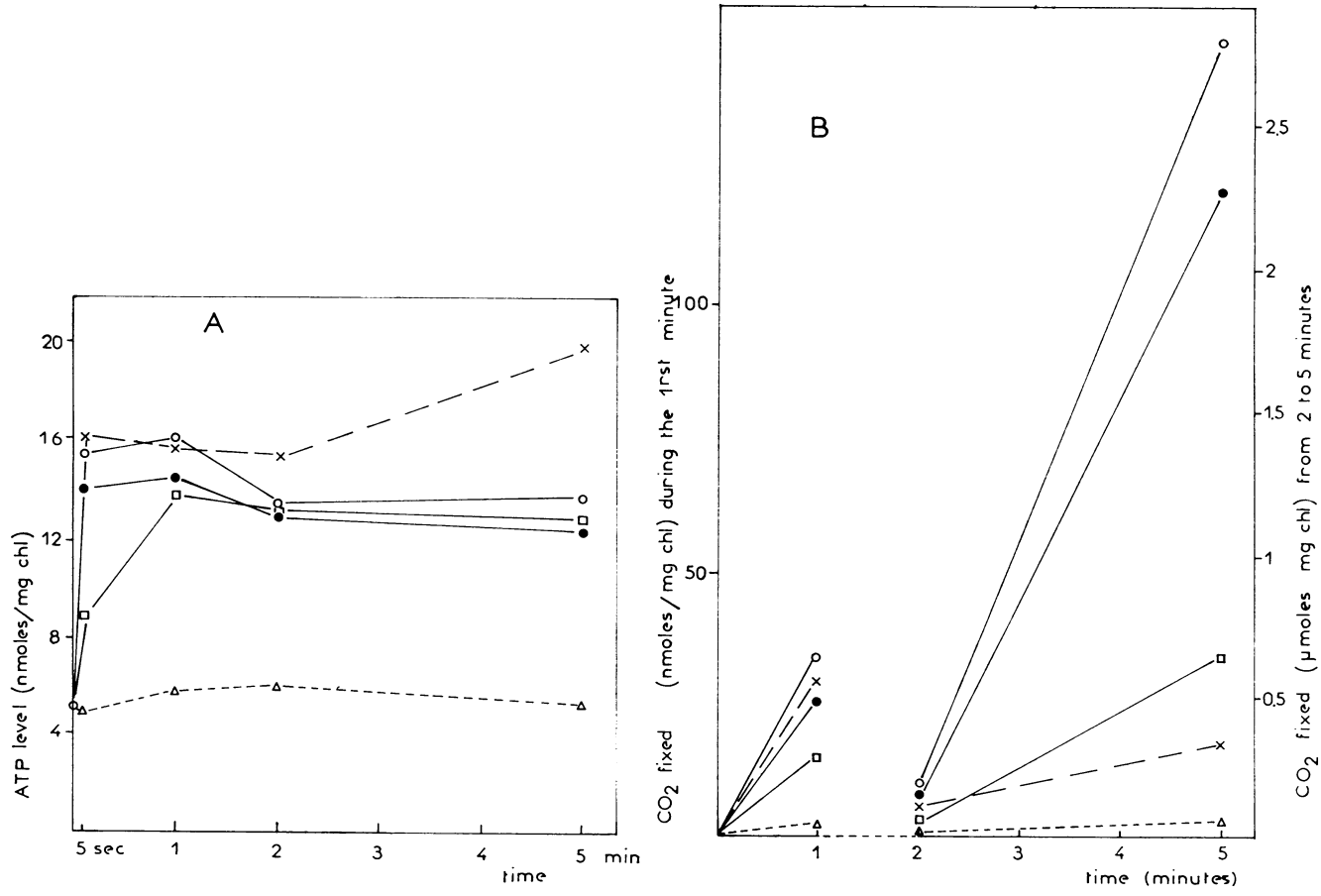

FIG. 2. Effect of antimycin A at various concentrations on the progress curve of CO_2 fixation and on the simultaneous changes of the ATP levels in illuminated spinach chloroplasts. Components of the reaction mixture were as described in the text under "Materials and Methods." Chl concentration was 112.5 μg/ml and antimycin A was at concentrations indicated. A: changes of the ATP level; B: progress curve of CO_2 fixation. Antimycin A treatments and rates of CO_2 fixation during 5 to 15 min intervals: control, 3.9 μmoles CO_2/mg Chl·hr (×); 1 μM Antimycin A, 44.7 μMoles CO_2/mg Chl·hr (○); 5 μM Antimycin A, 37.1 μmoles CO_2/mg Chl·hr (●); 25 μM Antimycin A, 14.8 μmoles CO_2/mg Chl·hr (□); 100 μM Antimycin A, 1 μmole CO_2/mg Chl·hr (△).

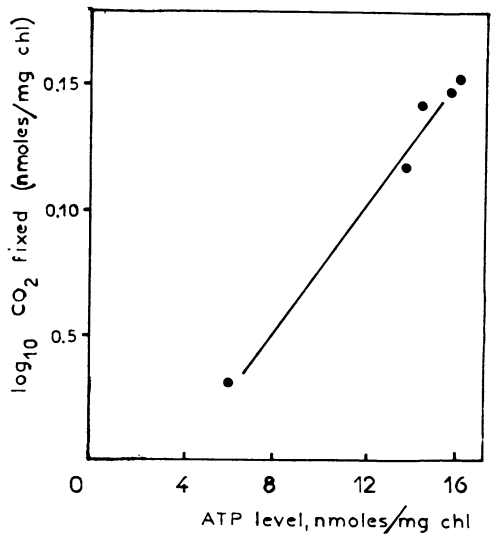

FIG. 3. Relationship between the amount of CO_2 fixed and the level of endogenous ATP of spinach chloroplasts after 1 min illumination. The data are from the experiment reported in Figure 2.

rates of carboxylation are always connected with lower levels of ATP and vice versa. However, the low level of ATP in the more active chloroplasts may be the direct consequence of the increased demand for ATP and the rate of ATP synthesis may be correspondingly greater (36). From the ^{14}C-products formed, it was calculated that the amount of ATP consumed by chloroplasts in the presence of antimycin is always much higher than the amount consumed by the control chloroplasts. Because of the higher rate of synthesis of phosphorylated compounds, the rate of ATP turnover is expected to be higher.

Although the steady state level of ATP is similar for the three concentrations of antimycin which stimulate the carboxylation activity (Fig. 2A), the rate of the ATP to ADP turnover depends on the rate of the CO_2 fixation. The more the carboxylation is increased, the more the turnover may be enhanced (Fig. 2B).

Table I shows the levels of ADP and AMP compared with ATP. They are in agreement with other estimations on isolated spinach chloroplasts (35). The presence of antimycin in the reaction mixture determines a higher level of ADP as a consequence of the lower level of ATP. This is especially evident after 2 or 5 min illumination.

The cumulative effect of ATP, ADP, and AMP can be investigated by the energy charge study defined as (ATP + ½ ADP)/(AMP + ADP + ATP). Atkinson (1) suggested energy charge to be a fundamental metabolic control parameter for enzymes utilizing ATP, and since Bomsel and Pradet (6) emphasized the regulatory importance of the adenylate kinase equilibrium system in plants, the energy charge regulation of chloroplast PGA kinase activity was demonstrated by Pacold and Anderson (32) and by Lavergne and Bismuth (unpublished data). However, after 2 min of photosynthesis, that is nearly at the end of the induction period, no relation is obvious between the energy charge and the CO_2 fixation activity, except for the 100 μM inhibiting concentration (Table II). During the steady state, the energy charge is lower in the antimycin-stimulated chloroplasts than in the control chloroplasts. The higher rate of carboxylation, although it consumes more energy, would not be dependent on the higher energy charge.

The sum (ATP + ADP + AMP) is not constant. It varies as a function of time. This fact has already been observed by other workers (5), but no definite explanation has yet been offered for it.

Products of CO_2 Fixation. The distribution of ^{14}C among several intermediates of the Calvin cycle has been studied in the same experiment as the kinetics of CO_2 fixation, and the changes in adenine nucleotides illustrated in Figures 1, 2, and 4, and in Table III.

Table I. *Effect of Antimycin A at Various Concentrations on the Changes of the Adenine Nucleotide Levels in Illuminated Spinach Chloroplasts*

Effects were measured during the induction period (1 and 2 min) and during the steady-state (5 min); effect on the ATP/ADP ratio is also shown. The results are from the same experiment as that reported in Figure 2. Components of the reaction mixture were as described in the text under "Materials and Methods" and antimycin A was at concentrations indicated. Chl concentration was 112.5 μg/ml.

Time of Illumination	Antimycin A Conc	Adenine Nucleotides Levels			ATP/ADP
		ATP	ADP	AMP	
min	μM	*nmoles/mg Chl*			*ratio*
0		5.1 ± 0.12	8.0 ± 0.66	9.6 ± 3	0.6
	0	15.6	4.6	5.0	3.4
	1	16.0	4.4	6.3	3.6
1	5	14.4	4.7	0	3.1
	25	13.7	5.6	9.2	2.4
	100	5.8	8.0	10.4	0.7
	0	15.2	3.2	1.0	4.7
	1	13.4	4.9	4.6	2.7
2	5	12.9	5.3	2.8	2.4
	25	13.1	6.3	1.2	2.1
	100	6.0	8.3	8.5	0.7
	0	19.5	3.0	0	6.5
	1	13.6	7.3	0	1.9
5	5	12.2	6.9		1.8
	25	12.6	7.9	0	1.6
	100	5.2	9.2	2.6	0.6

Table II. *Effects of Antimycin A at Various Concentrations on the CO_2 Fixation Activity during the Steady State and on the Energy Charge of Illuminated Spinach Chloroplasts*

Energy charge was measured at the end of the induction period (2 min) and during the steady state (5 and 10 min). Data are calculated from the results of the experiment reported on Table I. Components of the reaction mixture were as described in the text under "Materials and Methods" and antimycin A was at concentrations indicated. Chl concentration was 112.5 μg/ml. The dark value of energy charge in isolated chloroplasts is 0.455 ± 0.038. In three different experiments at 5 and 10 min illumination, the energy charge values of the control were always higher than those obtained in the presence of antimycin.

Antimycin A	Carboxylation Activity during the Steady State	Time of Illumination		
		2 min	5 min	10 min
μM	μmoles/mg Chl·hr	*energy charge*: (ATP + ½ ADP)/(ATP + ADP + AMP)		
0	3.9	0.75	0.94	0.92
1	44.7	0.70	0.83	0.76
5	37.1	0.74	0.69	0.81
25	14.8	0.78	0.80	0.85
100	1.0	0.45	0.73	0.68

After 1 minute of illumination the distribution of ^{14}C into the products is quite similar regardless of the amount of CO_2 fixed. Antimycin A causes only a small reduction in the percentage of fructose-6-P. The most striking observation at 2 min is the higher labeling of ribulose-1,5-diP and lower labeling of fructose-1,6-diP in the presence of antimycin as compared with the control. This is only momentary and it is clear, especially from the 25 μM antimycin experiment, that it precedes the enhancement of CO_2 fixation. It strongly suggests an activation of the hexose diPase and ribulose-5-P kinase which catalyze the transformation of fructose-1,6-diP into ribulose-1,5-diP, and which are known as light-regulated enzymes of the Calvin cycle (3, 26).

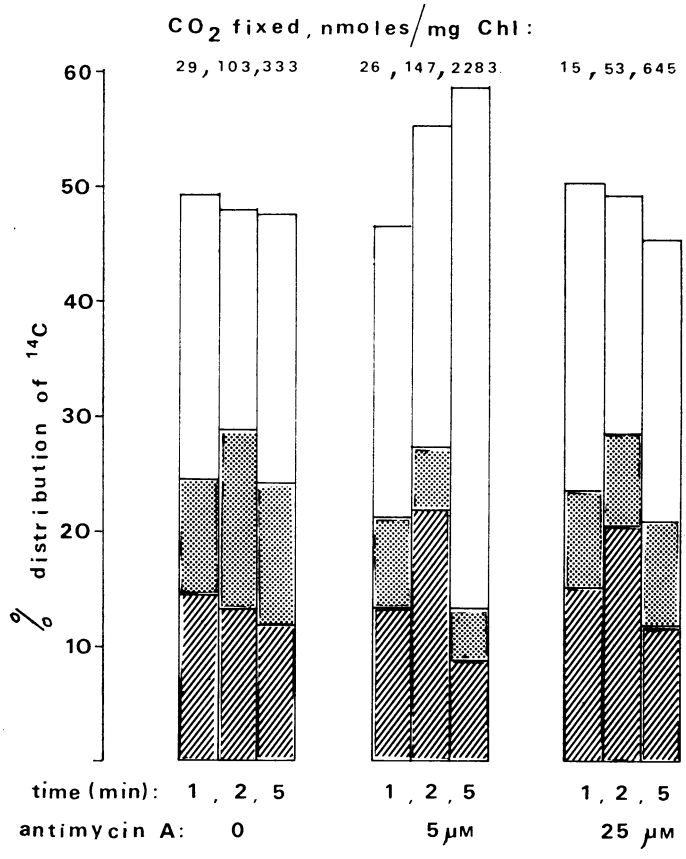

FIG. 4. Effect of antimycin A at various concentrations upon the distribution of ^{14}C in ribulose-1,5-diP, fructose-1,6-diP, and glycerate-3-P (graph drawn from the data of Table III). □: glycerate-3-P; ⊡: fructose-1,6-diP; ▨: ribulose-1,5-diP.

At 2 min, in the presence of 5 μM antimycin A, the induction phase of carboxylation is already completed and the enhancement of PGA synthesis is evident. In the presence of 25 μM antimycin A, the length of the induction period is longer than 2 min. The enhancement of the carboxylation activity is delayed if compared to the enhancement of ribulose-1,5-diP synthesis and the relative labeling of ribulose-1,5-diP drops with increased CO_2 fixation.

When comparing the PGA *versus* adenine nucleotide determinations, it can be seen that the PGA accumulation in the antimycin treated chloroplasts is concomitant with a lower ATP level and a lower energy charge as already presumed (11, 28, 29).

From our data we cannot determine whether the predominant factor of the C distribution between PGA and triose-P is the energy charge or the ATP/ADP ratio, but the existing evidence on the regulation of PGA-kinase by energy charge favors the former hypothesis.

CONCLUSION

This study demonstrates that in intact spinach chloroplasts, antimycin A inhibits photophosphorylation as it does in chloroplasts fragments, although it is not yet possible to claim whether it acts as a specific inhibitor of cyclic photophosphorylation, as an uncoupler, as an energy transfer inhibitor, or as a stimulant of the ATPase activity.

The antimycin-enhanced rate of the steady state carboxylation always follows a slight decrease in the ATP level resulting from the reduced rate of ATP synthesis during the induction period.

Table III. *Effect of Antimycin A at Various Concentrations upon the Amount of CO_2 Fixed and Products of Carboxylation by Illuminated Spinach Chloroplasts*

The results are from the same experiment reported in Figure 2. Components of the reaction mixture were as described in the text under "Materials and Methods" and antimycin A was at concentrations indicated. Chl concentration was 112.5 μg/ml.

Time of illumination	1 min						2 min						5 min					
Antimycin A concn (μM)	0		5		25		0		5		25		0		5		25	
Nanomoles CO_2 fixed per mg Chl	29		26		15		103		147		53		333		2 283		645	
	a[1]	b[2]	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Glycerate-3-P	7.3	24.7	6.3	24.3	4.1	26.6	19.6	18.9	41	27.9	11.9	22.5	77.8	23.4	1017	44.6	168	26.1
Triose-P[3]	10	33.9	9.4	35.9	5.5	35.3	42.1	40.8	40.5	30.9	21.1	39.9	131.7	39.5	742	32.5	265	41.1
Fructose-1,6-diP[4]	2.9	10	2.2	8.3	1.3	8.7	16.2	15.7	8.2	5.6	4.1	7.8	41.4	12.4	100	4.4	43	6.7
Fructose-6-P	2.4	8.1	1.5	5.8	0.7	4.7	6.3	6.1	14.9	10.2	3.8	7.1	21.7	6.5	105	4.6	50	7.7
Ribose-5-P	1	3.2	1.2	4.4	0.5	3.4	3.1	3.0	3.2	2.1	1	1.8	16.5	5.0	83	3.7	30	4.6
Ribulose-1,5-diP	4.4	14.9	3.6	13.8	2.4	15.5	14.2	13.8	32.5	22.1	10.1	19.1	39.8	12.0	213	9.3	80	12.4
Insoluble	1.5	5.2	2	7.5	0.9	5.8	1.8	1.8	1.7	1.2	1	1.8	4.1	1.2	21	0.9	9	1.4

[1] a: natoms C incorporated in the products.
[2] b: % distribution of ^{14}C in the products.
[3] Dihydroxyacetone-P and glyceraldehyde-3-P.
[4] With small amounts of sedoheptulose-1,7-diP.

The relationship between the CO_2 fixation activity of illuminated chloroplasts and their levels of adenylate nucleotides seems to be quite different during the induction period and the steady state. During the time of induction the amount of CO_2 fixed is exponentially related to the level of ATP. On the contrary, during the steady state the stimulation of CO_2 assimilation seems to be related to a slight decrease in the ATP level or in the ATP/ADP ratio during the previous induction, and then the activated rates of carboxylation are always concomitant with lower ATP levels. The rate of ATP turnover during the steady state is certainly higher in the presence of antimycin, because it depends on the activities of CO_2 fixation and phosphorylated sugar synthesis which provide ADP. Indeed, the rate of ATP synthesis is controlled entirely by the ADP supply and by the photophosphorylation capacity which is more or less lowered depending on the concentration of antimycin. Very likely the former factor seems to be the most determinant.

The kinetic analysis of the ^{14}C-labeling of the products of carboxylation seems to indicate that the primary effects of antimycin induce a gradual stimulation of the Calvin cycle steps. The activation of ribulose-1,5-diP generation during the induction period precedes the activation of the carboxylation reaction, which happens at the beginning of the steady state.

In an attempt to resolve the means by which the effect of antimycin on the decrease in the ATP/ADP ratio during the induction period can induce the ultimate stimulation of the carboxylation activity, an alternative hypothesis may be put forward: because the photosynthetic carboxylation enhancement which follows the decrease in the ATP/ADP ratio denotes the activation of the photochemical electron transfer, it is feasible that either a photosynthetic control or a partial uncoupling occurs, insuring a higher NADP reduction rate.

The link between the antimycin-dependent decrease in the ATP/ADP ratio and the activation of the enzymes may be visualized as a consequence of the electron transfer activation hypothesis, through the ΔpH and the Mg^{2+} concentration of the stroma; the working hypotheses are summarized in Figure 5.

It is known that the CO_2 fixation activity of intact chloroplasts is favored by the enhancement of ΔpH between the stroma and the thylakoid compartments (21). An increase of proton absorption into the thylakoid space may result from an energy-transfer inhibition or from a stimulation of the ATPase activity (9, 15, 25). In case of uncouplers, a decrease in the light-induced ΔpH has been reported several times (15, 21, 39), except for one particular compound which increased it (14).

As a consequence of the ΔpH increase, the Mg^{2+} concentration of the stroma is higher and its alkalization would enable the concentration of bicarbonate which has been established by Werdan *et al.* (42), to be inversely proportional to the proton distribution.

Increase of pH, increase of Mg^{2+} level (16, 30, 31), and concentration of CO_2 (as bicarbonate) in the stroma compartment are three factors which markedly stimulate the activity of some enzymes of the pentose-phosphate reduction cycle: the stimulation of fructose diphosphatase at pH 7.8 and by Mg^{2+} concentration is now well established (19, 34). It was reported that the phosphoribulokinase is very responsive to Mg^{2+} activation (22,

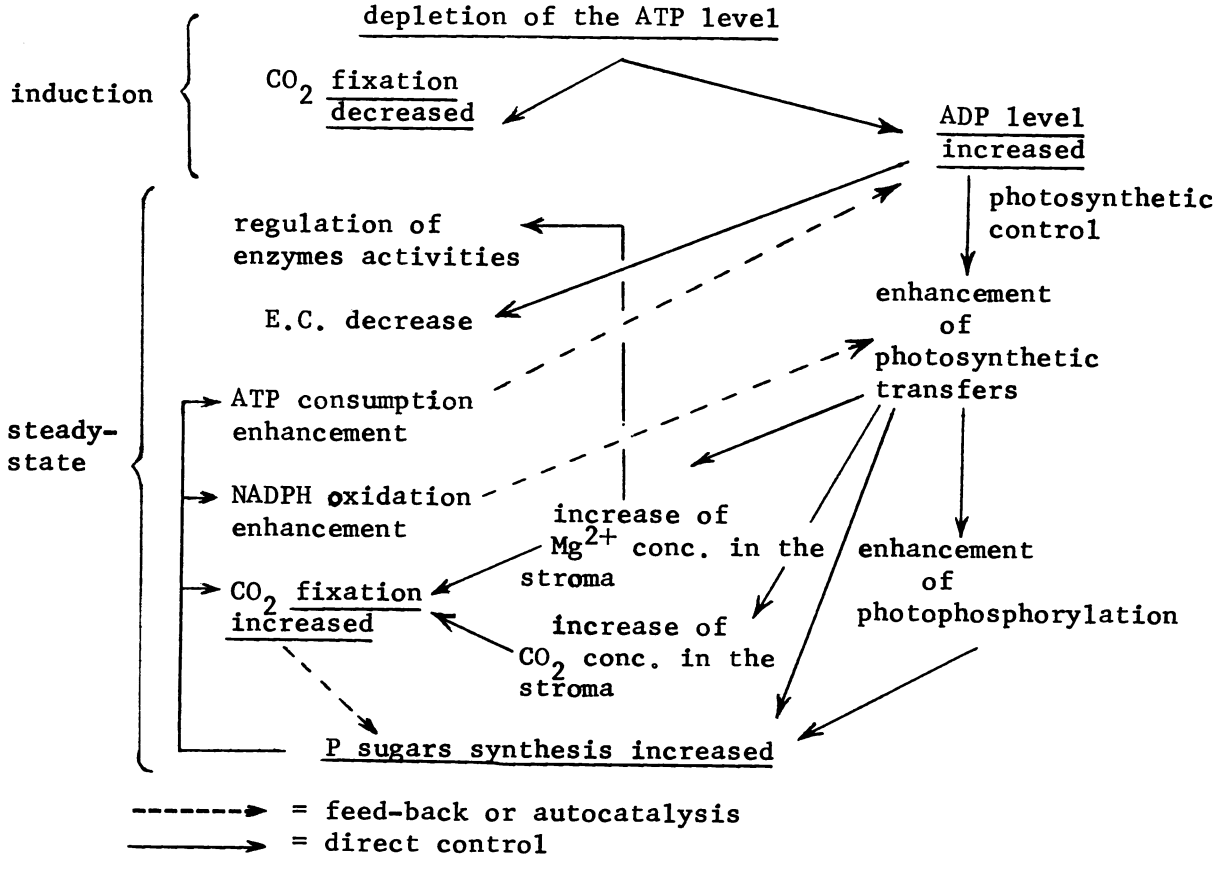

FIG. 5. Summary of the hypothesis which may be considered to link the effect of antimycin A on the adenine nucleotides level during the induction period and the ultimate enhancement of the steady state carboxylation activity of spinach chloroplasts E.C. = energy charge. Underlined are the results already demonstrated and basis of the working hypotheses.

24). Ribulose-1,5-diP carboxylase also requires Mg^{2+} for full activity and is most active at about pH 7.6 (4).

Indeed the stimulation of ribulose-1,5-diP synthesis from fructose-1,6-diP before the end of the induction period would favor the hypothesis of the enhancement of ΔpH in the chloroplasts and would affect the activation of fructose diphosphatase and ribulose-5-P kinase. The delay of the enhancement of the carboxylation activity after the stimulation of ribulose-1,5-diP synthesis suggests that either the increase of the ribulose-1,5-diP level or the decrease of fructose-1,6-diP, which is inhibitory to the ribulose-1,5-diP carboxylase (8), or both together, might be the primary factors in the stimulation of the carboxylation reaction. This assumption does not exclude the possibility of a direct activation of the ribulose diphosphate carboxylase by modified pH and high level of Mg^{2+} in the stroma. Then the fructose diphosphatase and perhaps also the ribulose-5-P kinase would be the pacesetting enzymatic steps of the CO_2 fixation activity, as proposed by Jensen (24) and Heldt *et al.* (21). Furthermore the increased cycling of phosphorylated sugar synthesis would contribute to the stimulation of the carboxylation activity in an autocatalytic process by generating NADP and ADP.

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