Plant Physiol. (1971) 48, 707-711

Involvement of Photosynthetic Carbon Reduction Cycle Intermediates in CO_2 Fixation and O_2 Evolution by Isolated Chloroplasts[1]

Received for publication May 19, 1971

BERNICE SCHACTER,[2] J. H. ELEY,[3] AND MARTIN GIBBS
Department of Biology, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

The photosynthetic carbon reduction cycle intermediates can be divided into three classes according to their effects on the rate of photosynthetic CO_2 evolution by whole spinach (*Spinacia oleracea*) chloroplasts and on their ability to affect reversal of certain inhibitors (nigericin, arsenate, arsenite, iodoacetate, antimycin A) of photosynthesis: class I (maximal): fructose 1,6-diphosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, ribose-5-phosphate; class 2 (slight): glucose 6-phosphate, fructose 6-phosphate, ribulose-1,5-diphosphate; class 3 (variable): glycerate 3-phosphate. While class 1 compounds influence the photosynthetic rate, they do not lower the Michaelis constant of the chloroplast for bicarbonate or affect strongly other photosynthetic properties such as the isotopic distribution pattern. It was concluded that the class 1 compounds influence the chloroplast by not only supplying components to the carbon cycle but also by activating or stabilizing a structural component of the chloroplast.

Intact chloroplasts, in contrast to fragmented chloroplasts, will evolve oxygen and reduce carbon dioxide to carbohydrate (17). Some, but not all, intermediates of the photosynthetic carbon reduction cycle are known to stimulate photosynthesis in the intact chloroplast (4, 5). It has also been shown that intermediates such as fructose 1,6-diP and ribose 5-P which can stimulate photosynthesis of the isolated chloroplast, in contrast to glucose 6-P which has little effect, can restore carbon dioxide assimilation or oxygen evolution inhibited by arsenite (4), iodoacetamide (4), or orthophosphate (8) but not by DCMU (3) or 2-heptyl-4-hydroxyquinoline-N-oxide (3).

The purpose of the present study was to characterize more fully the stimulation of photosynthesis by components of the photosynthetic carbon reduction cycle with emphasis on their ability to reverse inhibitors specific for either the carbon cycle or the photochemical reactions. A preliminary description of this research has been published (15).

[1] This research was generously supported by the National Science Foundation.

[2] Present address: Department of Chemical Biodynamics, Lawrence Radiation Laboratory, Berkeley, Calif. 94720.

[3] Present address: Department of Botany, University of Kentucky, Lexington, Ky. 40506.

MATERIALS AND METHODS

Intact chloroplasts were prepared from *Spinacia oleracea* by the method of Jensen and Bassham (12). Five grams of leaves from the greenhouse, environmental room, or obtained locally in the field, were deribbed, cut into small pieces, and subsequently placed in a semimicro homogenizing vessel and blended for 5 sec with 25 ml of solution A containing MES buffer, pH 6.1. The homogenate was filtered through cheesecloth and centrifuged at 2000*g* for 50 sec. The resulting pellet was resuspended in approximately 2 ml of solution B containing HEPES buffer, pH 6.7. The addition of 0.1 ml of this suspension containing intact chloroplasts to 2.9 ml of reaction mixture gave a chlorophyll concentration of from 20 to 40 μg/ml as determined by the method of Arnon (1). The reaction mixture for both CO_2 and O_2 measurements contained 0.33 M sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 5 mM $Na_4P_2O_7$, and 50 mM tricine, pH 8.1. In addition, the reaction mixture for measurement of CO_2 uptake usually contained 15 μmoles of $NaHCO_3$, containing approximately 25 to 75 μc of ^{14}C, while that for O_2 evolution contained 15 μmoles of unlabeled $NaHCO_3$. Concentration of other components is given in the text.

Oxygen evolution was measured polarographically with a modified Clark-type electrode inserted into a 20 C thermostatted Lucite chamber containing the reaction mixture. Prior to being put into the chamber, the reaction mixture was bubbled with N_2. The sensitivity of the polarographic apparatus allowed operation with a full scale deflection equal to a change in O_2 concentration of 20 μM. Light was provided by a DWY tungsten-iodine lamp with infrared radiation removed by a combination of infrared-absorbing glass and a 5 cm water filter. Intensity was controlled by precalibrated screens and measured with a Weston Model 756 illumination meter. The rate of O_2 evolution was calculated from the highest linear rate after correction for dark uptake.

The rate of CO_2 fixation was measured by $^{14}CO_2$ uptake in test tubes bubbled with N_2 at 20 C. Illumination was provided by four 150-w flood lamps adjusted by lamp voltage to provide light intensity comparable to that used for oxygen evolution measurements. At known intervals, aliquots were removed, and the reaction was stopped with formic acid. After drying, the amount of isotope fixed was determined with a gas flow thin window counter. Fixation rates were calculated from that portion of the time course which gave the highest linear rate.

RESULTS

General. Bamberger and Gibbs (4) utilized spinach chloroplasts isolated in sodium chloride, whereas in the present study

Table I. *Effect of Some Intermediates of the Photosynthetic Carbon Reduction Cycle on the Distribution of $^{14}CO_2$ Assimilated*

Incubation was carried out in the standard reaction mixture under N_2 with $NaHCO_3$ at 5 mM. The sugar phosphates were added as indicated to give a concentration of 1 mM. The samples used for paper chromatography were taken 10 min after turning on the light. Chromatography was carried out as described in references 14 and 16.

	Control	Fructose-1,6-diP	Ribose-5-P	Fructose-6-P
	% total cpm fixed			
Insolubles + sugar diphosphates[1]	16.7	17.5	21.0	15.9
Sugar monophosphates[2]	5.1	4.4	7.7	16.4
Glycerate 3-P	14.1	17.3	21.7	21.1
Triose phosphates[3]	56.3	57.5	46.2	42.9
Glycolate	8.0	2.9	3.4	3.7
Rate of fixation μmoles/mg chl·hr	42	60	64	42

[1] Polyglucan, ribulose diphosphate, and fructose diphosphate.
[2] Glucose and fructose.
[3] Essentially dihydroxyacetone-P, some glyceraldehyde 3-P.

and in that of Bucke *et al.* (5), chloroplasts were prepared in sorbitol. From these investigations, a general pattern arises that the photosynthetic carbon cycle intermediates can be divided into three classes according to their effects on the rate of photosynthetic CO_2 fixation or O_2 evolution and on their ability to affect reversal of certain inhibitors of photosynthesis:

Class I. Maximal stimulation and reversal but, on occasion, had no effect: fructose 1,6-diP, dihydroxyacetone-P, glyceraldehyde 3-P, ribose 5-P.

Class II. Effect slight: glucose 6-P, fructose 6-P, ribulose 1,5-diP.

Class III. Stimulation and reversal variable depending on the preparation: glycerate 3-P.

Table I shows the effect of a number of these compounds on the distribution of isotope during photosynthesis in $^{14}CO_2$. Fructose 6-P did not alter the rate of fixation but did affect the location of isotope.

Stimulation under Limiting Conditions. In order to determine whether fructose 1,6-diP could increase the rate of photosynthesis under bicarbonate limiting, as well as saturating conditions, the experiment recorded in Figure 1 was done. A plot of S/V *versus* S is given, and the line of best fit determining the *Km* was obtained by method of least squares. The *Km* for bicarbonate was approximately 1 mM and appeared to be unchanged by fructose 1,6-diP, although the rate of fixation was increased at all bicarbonate concentrations used.

Photosynthesis, as measured by O_2 evolution or CO_2 fixation, was also stimulated by fructose 1,6-diP when light was limiting. For example, at 300 ft-c, chloroplasts evolved 12 μmoles O_2/mg chl·hr, whereas the rate was doubled on addition of 1 mM fructose 1,6-diP. When the light intensity was tripled, the O_2 value increased to 20 μmoles/mg chl·hr and, on addition, of 1 mM fructose 1,6-diP, a rate of 35 was observed.

Inhibitors of the Carbon Cycle. Compounds listed in class I can reverse the inhibition of CO_2 fixation caused by arsenite (4) and iodoacetic acid (4). Figures 2 and 3 show the effect of glycerate 3-P and ribose 5-P on photosynthesis in the presence of a strongly inhibitory concentration of iodoacetic acid. Clearly, in the presence of these carbon cycle intermediates,

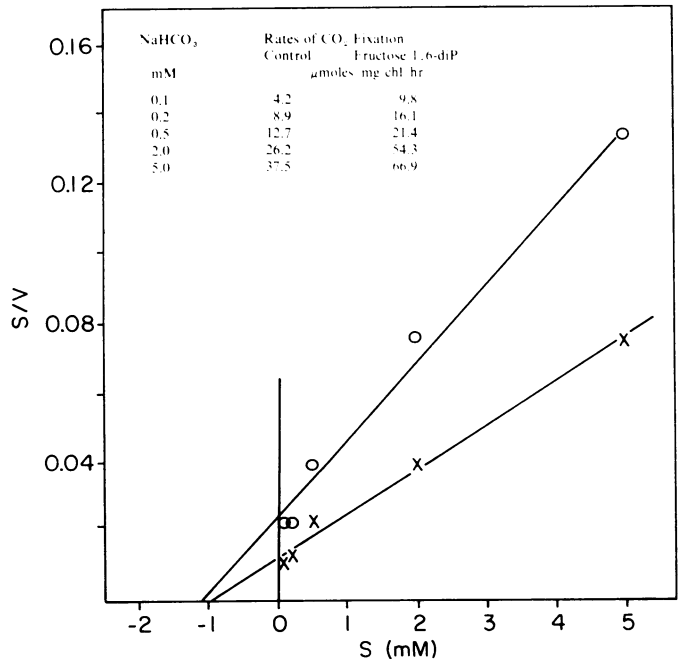

FIG. 1. *Km* (bicarbonate) of chloroplasts. S/V *versus* S plot for rates of CO_2 fixation in the presence and absence of 1 mM fructose 1,6-diP in response to varying concentrations of $NaHCO_3$. Incubation was in the standard reaction mixture under N_2 with a light intensity of 2600 ft-c. Rates of fixation were determined during the 2 min to 6 min interval after the light had been turned on, the period which gave the highest linear rate for each concentration of $NaHCO_3$. Control (×) and fructose 1,6-diP (○).

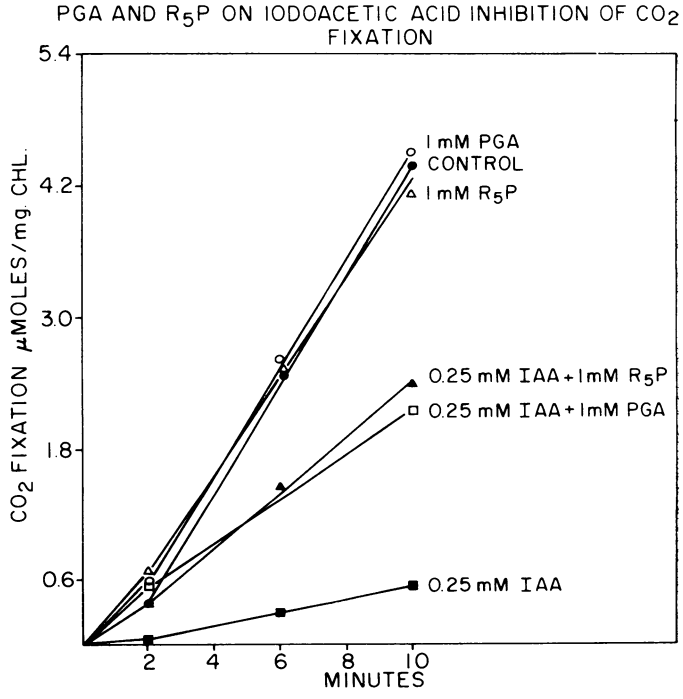

FIG. 2. Effect of glycerate 3-P and ribose 5-P on inhibition of CO_2 fixation by iodoacetic acid. Incubation was in the standard reaction mixture for CO_2 fixation with $NaHCO_3$ at 5 mM, under N_2 and with an illumination of 2600 ft-c. Rates of fixation in μmoles/mg chl·hr were: control, 29 (●); ribose 5-P (R_5P), 28 (△); glycerate 3-P (PGA), 33 (○); iodoacetic acid (IAA), 3 (■); iodoacetic acid + ribose 5-P, 16 (▲); iodoacetic acid + glycerate 3-P, 15 (□).

the rates of both CO_2 fixation and O_2 evolution were partially restored.

In an experiment where the rate of CO_2 fixation was followed in the presence of 0.25 mM iodoacetic acid, there were only relatively small changes in the distribution pattern recorded in Table I in contrast to the marked inhibition (90%) of the rate (data not given). The presence of 1 mM glycerate 3-P reduced the inhibition to 50% with little effect on the products formed. The one major change was a shift of isotope from glycerate 3-P to triose-P in the presence of iodoacetic acid and similarly in the presence of iodoacetic acid and glycerate 3-P. A decrease in glycolate was also observed.

Uncouplers of Photophosphorylation. Compounds of class I reversed the inhibitions of CO_2 fixation and O_2 evolution by arsenate (Fig. 4), chlorocarbonyl cyanide phenylhydrazone, and its trifluoro derivative, desaspidin, and nigericin (data of the latter not shown).

Antimycin A. Antimycin A stimulates the rate of CO_2 fixation and O_2 evolution in intact chloroplasts (7). It was, therefore, of interest to determine whether the two stimulatory effects were related. In a typical experiment the rate of CO_2 fixation was stimulated about 2-fold by fructose 1,6-diP and 3-fold by the antibiotic. The combination of the two increased the rate by a factor of roughly 4-fold (data not shown). Thus, the effects seem additive.

Effect of Photosynthetic Carbon Cycle Intermediates on Partial Reactions of Photosynthesis. In an effort to determine whether the stimulation of the rate of photosynthesis by intact chloroplasts was related to reducing power, the effect of fructose 1,6-diP on TPN reduction and $^{32}P_i$ esterification was measured in chloroplast fragments. Fructose 1,6-diP (1 mM) had no effect on these reactions. Furthermore, 1 μM trifluorocarbonyl cyanide phenylhydrazone inhibited photophosphorylation by 30% and stimulated electron flow by 30%, but 1 mM fructose 1,6-diP did not reverse these reactions in fragmented chloroplasts (data not shown).

DISCUSSION

Stimulation under Limiting Conditions. We interpret our findings to indicate that some intermediates of the photosynthetic carbon reduction cycle can bring about the potential ability of isolated chloroplasts to carry out photosynthesis. The basis for this suggestion is the ineffectiveness of fructose 1,6-diP to alter the affinity of the chloroplasts for $NaHCO_3$ (Fig. 1) and the absence of a change in light intensity needed for saturation (see "Results" and [14]). We conclude that during photosynthesis limited by bicarbonate or light, the addition of fructose 1,6-diP affects the rate because it increases the V_{max}, not because it lowers the Km. The Km for bicarbonate remains the same but more active chloroplasts are apparent in the assay. These units with identical Km contribute to the observed fixation rate. The activation of photosynthesis noncompetitive with respect to CO_2 by intermediates of the photosynthetic carbon reduction cycle indicates that structural modifications of proteins may be occurring.

Antimycin A at low concentrations (5 μM) stimulates photosynthesis in chloroplasts when CO_2 but not light is limiting (16). The data presented in "Results" are indicative that the effect of the antibiotic differed from that of the class I intermediates. Based on a lowering of the affinity of the chloroplast for bicarbonate, the speculation was presented that antimycin A facilitated in some manner the primary carboxylation reaction (16). In contrast, intermediates of the photosynthetic carbon reduction cycle do not seem to influence any specific site in the chloroplast but rather have a general effect.

Inhibitors. The significance of the inhibitor studies on CO_2 fixation and accompanying O_2 evolution must be considered in terms of the variety of inhibitors used and the consistency of the response. The general conclusions of the inhibitor studies are two: (*a*) any moderate block by inhibitors of the photosynthetic carbon reduction cycle or by uncouplers of the electron transport chain, as measured in intact chloroplasts in terms of

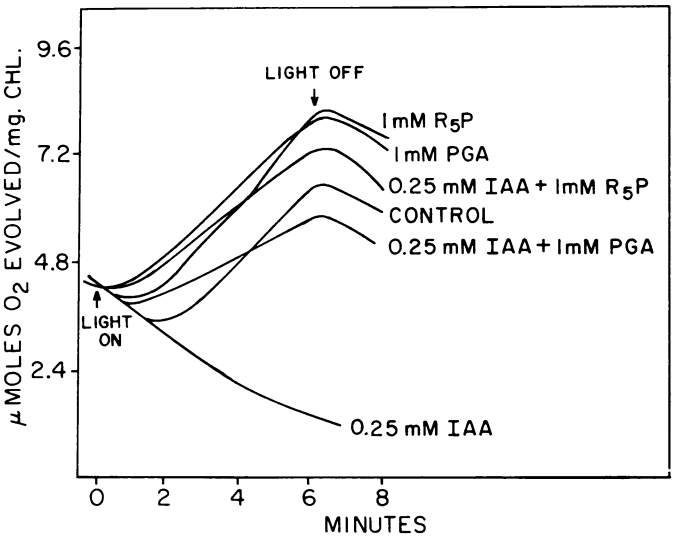

FIG. 3. Effect of ribose 5-P and glycerate 3-P on the inhibition of O_2 evolution by iodoacetic acid. Incubation was in the standard reaction mixture, with $NaHCO_3$ at 5 mM, under N_2 and with an illumination of 2600 ft-c. Rates of O_2 evolution in μmoles/mg chl·hr were: control, 29; ribose 5-P (R_5P), 30; glycerate 3-P (PGA), 29; iodoacetic acid (IAA), 2; iodoacetic acid + ribose 5-P, 21; iodoacetic acid + glycerate 3-P, 30.

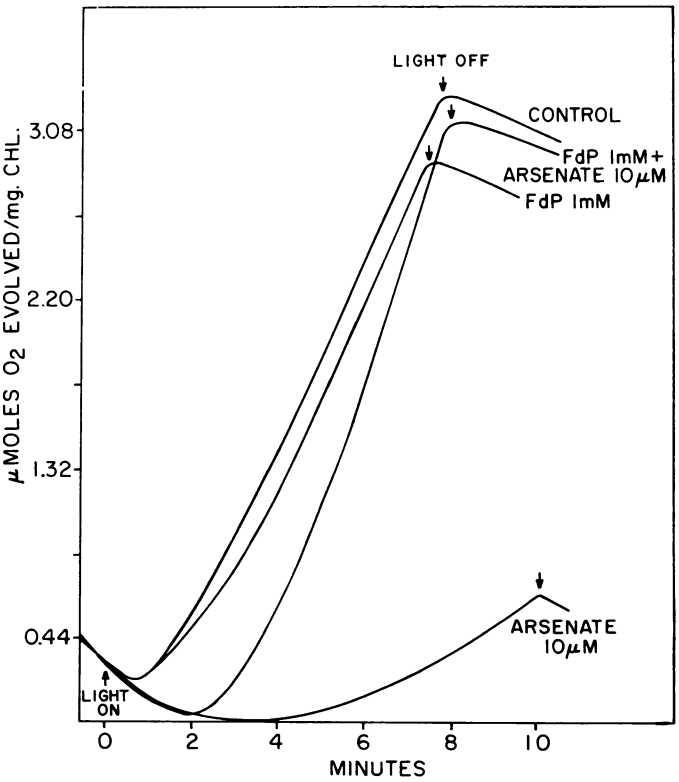

FIG. 4. Effect of fructose 1,6-diP on inhibition of O_2 evolution by arsenate. Incubation was in the standard reaction mixture under N_2 with $NaHCO_3$ at 5 mM, and light intensity of 2600 ft-c. Rates of O_2 evolution in μmoles/mg chl·hr were: control, 33; fructose 1,6-diP (FdP), 35; arsenate, 16; and arsenate + fructose 1,6-diP, 32.

CO_2 fixation or O_2 evolution, was restored by class I compounds, regardless of the site or manner of action of the inhibitor used; (*b*) the photosynthetic quotient in the chloroplast is roughly one (Figs. 2, 3) and an inhibition of one parameter, O_2 evolution or CO_2 fixation, resulted in an inhibition of the other.

Iodoacetic acid (1 mM) and arsenite (1 mM) have little effect upon the energy conversion activities in fragmented chloroplasts (6, 10). Iodoacetic acid (6) is known to inhibit ribulose 5-P kinase and glyceraldehyde 3-P dehydrogenase. On the other hand, arsenite is thought to block carbon metabolism at the ribulose 5-P kinase step (10). Our data show that the inhibition by both inhibitors was reversed by class I intermediates. Not only was CO_2 fixation restored, but, more importantly, a photosynthetic quotient of unity was recorded. The restoration of O_2 evolution and the observation that the usual pattern of distribution of radioisotope within the Calvin-Benson cycle intermediates reappears is taken as evidence that the chloroplast was functioning normally. Earlier, when CO_2 assimilation was the only parameter of photosynthesis measured, it was thought that the class I intermediates bypassed the enzymic block by furnishing carbon skeletons (4, 5). Clearly, this conclusion must now be modified.

On the basis of the well established irreversible inhibition of sulfhydryl enzymes by iodoacetic acid and arsenite, it is difficult to envisage a definitive mechanism whereby the class I compounds restore photosynthesis. We propose the following hypothesis. Most likely the sulfhydryl-containing enzymes of the chloroplast are in a mixed state of oxidation, partly in the -SH and partly in the S-S form. The former, but not the latter, would be alkylated by the inhibitor. The addition of a class I intermediate would flood the chloroplast with an excess of Calvin-Benson cycle intermediates, such as glyceraldehyde 3-P and ribulose 5-P, which in turn, could protect the -SH form resulting from the reduction of the -S-S- enzyme when illumination was begun. Supporting this mechanism of reversal are the following observations: (*a*) leaf tissue extract can reduce protein S-S groups to protein SH groups (11), however, the location of this TPN-linked enzyme has not been determined; (*b*) ribulose 5-P can protect purified ribulose 5-P kinase against inactivation by arsenite (13); (*c*) activation of ribulose 5-P kinase during illumination presumably as the result of the conversion of the S-S to SH form of the enzyme (13); (*d*) inhibition was not observed when iodoacetamide or arsenite was added during photosynthesis.

The uncouplers, chlorocarbonyl cyanide phenylhydrazone and its trifluoro derivative, nigericin, arsenate and desaspidin, used in this study differ in their properties and probable sites of action (2). Despite these differences, the inhibitions of CO_2 fixation and O_2 evolution of the isolated intact chloroplast caused by all uncouplers were relieved by class I compounds (Fig. 4 and [14]). This suggests that the relief was not site specific.

Similar to the stimulation of photosynthesis by antimycin A (16), reversal of the uncouplers by class I compounds was not observed in osmotically shocked chloroplasts. Evidently this characteristic of class I compounds required a chloroplast containing its outer membrane and once again, points out intrinsic differences in the properties of the two kinds of preparations.

The "Effective" Compound. With the initial observation of Bamberger and Gibbs (4) of the stimulatory effect of certain photosynthetic carbon cycle intermediates on CO_2 fixation by isolated chloroplasts, the question was raised of the actual compound or compounds responsible for this effect. The observations reported here do not add much to the answer of this question, however, relevant points can be made.

In general, the distribution pattern obtained under stimulatory conditions by class I compounds are similar enough to the control patterns to suggest that the effect of the carbon compounds is not a specific one and cannot be explained on the basis of the build-up of a particular intermediate. In some cases, addition of a class I compound tended to favor labeling of the added or related compounds. For example, fructose 1,6-diP usually increased the percentage of isotope in sugar diphosphates, but this could simply be related to a "pooling" or "trapping" of isotope by the unlabeled compound. The compounds that stimulated photosynthesis must have penetrated the chloroplast envelope, since U-^{14}C-fructose 1,6-diP was rapidly metabolized into the same products found with $^{14}CO_2$ (14). Unexpectedly, iodoacetic acid or uncouplers did not shift the distribution pattern, suggesting that no particular metabolic step was affected sufficiently to allow accumulation of isotope in an individual compound (14).

The possibility that class I compounds are involved in transphosphorylation, *i.e.*, between fructose 1,6-diP and ribulose 5-P, has been raised (9). It would seem that the clear lack of marked changes in ^{14}C distribution (Table I) and the fact that fructose 1,6-diP and ribose 5-P did not spare the esterification of inorganic phosphate ($^{32}P_i$) would lessen the possibility of transphosphorylation (14).

There is little doubt that class I compounds stimulate photosynthesis to some extent during the lag period by supplying intermediates to the photosynthetic carbon reduction cycle. But they must serve another function to explain stimulation of photosynthesis when light and bicarbonate are limiting and to explain the mechanism of reversal. We would propose that class I compounds stimulate the rate by activating or stabilizing a component of the chloroplast. That this compound is related to the structural integrity of the chloroplast seems likely from the insensitivity of the broken chloroplasts.

Cockburn *et al.* (8) have obtained parallel results for photosynthesis in chloroplasts with high concentrations of inorganic phosphate and class I compounds. Their report supports the generality of the effects with inhibitors reported here.

The exceptions to the general case of inhibition reversal by carbon cycle intermediates are those inhibitions of electron flow brought about by DCMU and 2-heptyl-4-hydroxyquinoline-N-oxide (3). These exceptions indicate that inhibitors affecting enzymes of carbon metabolism or ATP formation can be reversed but that the inhibitors of photosynthetic electron flow cannot be "reached" by class I compounds. This may reflect a structural impenetrability of the site. On the other hand, we have found that inhibition of CO_2 fixation and O_2 evolution by DCMU can be reversed by ascorbate. Furthermore, ascorbate was able to overcome the DCMU inhibition as well as increase the rate of photosynthesis at all light intensities.

LITERATURE CITED

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Avron, M. and J. Neumann. 1968. Photophosphorylation in chloroplasts. Annu. Rev. Plant Physiol. 19: 137–166.
3. Bamberger, E. S., C. C. Black, C. A. Fewson, and M. Gibbs. 1963. Inhibitor studies on carbon dioxide fixation, adenosine triphosphate fixation, and triphosphopyridine nucleotide reduction by spinach chloroplasts. Plant Physiol. 38: 483–487.
4. Bamberger, E. S. and M. Gibbs. 1965. Effect of phosphorylated compounds and inhibitors on CO_2 fixation by intact spinach chloroplasts. Plant Physiol. 40: 919–926.
5. Bucke, C. W., D. A. Walker, and C. W. Baldry. 1966. Some effects of sugars and sugar phosphates on carbon dioxide fixation by isolated chloroplasts. Biochem. J. 101: 636–641.
6. Calo, N. and M. Gibbs. 1960. The site of inhibition of iodoacetamide in photosynthesis studied with chloroplasts and cell-free preparations of spinach. Z. Naturforsch. 15b: 287–291.

7. CHAMPIGNY, M. L. AND M. GIBBS. 1970. Photosynthesis by isolated chloroplasts in the presence of antimycin A and ascorbic acid. *In:* H. Metzner, ed., Progress in Photosynthesis Research, Vol III. Tubingen City, pp. 1534–1537.
8. COCKBURN, W., C. W. BALDRY, AND D. A. WALKER. 1967. Some effects of inorganic phosphate on O_2 evolution by isolated chloroplasts. Biochim. Biophys. Acta 143: 614–624.
9. GIBBS, M. 1966. Carbohydrates: Their role in plant metabolism and nutrition. *In:* F. C. Steward, ed., Plant Physiology, A Treatise, Vol. IV B. Academic Press, New York. pp. 3–101.
10. GIBBS, M. AND N. CALO. 1960. Studies on carbon dioxide fixation by chloroplasts. The effect of arsenite and other factors. Biochim. Biophys. Acta 44: 341–347.
11. HATCH, M. D. AND J. F. TURNER. 1960. The mechanism of the reversible aerobic inhibition of glycolysis in a pea-seed extract. Biochem. J. 75: 66–72.
12. JENSEN, R. G. AND J. A. BASSHAM. 1966. Photosynthesis by isolated chloroplasts. Proc. Nat. Acad. Sci. U.S.A. 56: 1095–1101.
13. LATZKO, E., R. GARNIER, AND M. GIBBS. 1970. Effect of photosynthesis, photosynthetic inhibitors and oxygen on the activity of ribulose 5-phosphate kinase. Biochem. Biophys. Res. Commun. 39: 1140–1144.
14. SCHACTER, B. Z. 1970. Studies on the control of photosynthesis in spinach chloroplasts. Ph.D. thesis. Brandeis University, Waltham, Mass.
15. SCHACTER, B., J. H. ELEY, JR., AND M. GIBBS. 1968. Effect of sugar phosphates and photosynthesis inhibitors on CO_2 fixation and O_2 evolution by chloroplasts. Plant Physiol. 43: S30.
16. SCHACTER, B. Z., M. GIBBS, AND M. L. CHAMPIGNY. 1971. Effect of antimycin A on photosynthesis of intact spinach chloroplasts. Plant Physiol. 48: 443–446.
17. WALKER, D. A. AND A. R. CROFTS. 1970. Photosynthesis. Annu. Rev. Biochem. 39: 389–428.