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Photosynthesis by Isolated Chloroplasts

XII. INHIBITORS OF CO_2 ASSIMILATION IN A RECONSTITUTED CHLOROPLAST SYSTEM

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A previous article (1) has described a reconstituted "catalytic" chloroplast system in which, as in the intact leaf, the dark reactions of photosynthetic CO_2 assimilation depended on light for the generation of assimilatory power (2), *i.e.* reduced triphosphopyridine nucleotide and adenosine triphosphate. The companion paper (3) discusses the sites of action of photochemically generated ATP and $TPNH_2$ in the reductive carbohydrate cycle which operates in isolated chloroplasts. This communication gives further evidence for the action of the photochemically generated assimilatory power on two phases of the reductive carbohydrate cycle in isolated chloroplasts: the carboxylative phase which includes the phosphorylation of ribulose monophosphate and the fixation of CO_2, and the reductive phase, which includes the reduction of 3-phosphoglyceric acid and the formation of hexose phosphate. The evidence to be presented here is derived from experiments in which the availability of ATP and $TPNH_2$ for CO_2 assimilation was decreased either by the use of inhibitors or by special arrangements of experimental conditions; certain of the inhibitors acted on single enzymatic reactions in CO_2 assimilation.

METHODS

The methods used (including the preparation of ribulose diphosphate) were those described in the companion article (3). Further details are given in the tables and the legends to the figures.

RESULTS AND DISCUSSION

Inhibition of Carboxylative and Reductive Phases of CO_2 Assimilation through Shortage of ATP

Since ATP is required for the formation of ribulose diphosphate in the carboxylative phase and the formation of 1,3-diPGA[1] in the reductive phase (3), a shortage of ATP should inhibit both phases of CO_2 assimilation. Experimentally, a shortage of ATP was brought about in two ways: (1) by the use of uncouplers and inhibitors of photophosphorylation, or (2) by allowing ATP to form, but decreasing its availability for CO_2 assimilation through competition with another ATP-consuming enzyme system, hexokinase-glucose. The inhibition of the carboxylative phase was measured by a decrease in total CO_2 fixation, the inhibition of the reductive phase, by the preponderance of PGA among the products of CO_2 assimilation.

Uncoupling of ATP Formation from TPN Reduction by Ammonia—Krogman *et al.* (4) have discovered that in noncyclic photophosphorylation (5) with ferricyanide, ammonia effectively uncouples ATP formation from the photochemical reduction of ferricyanide. We have investigated the effect of ammonia when ATP was being formed simultaneously by cyclic photophosphorylation (Equation 1) and by noncyclic photophosphorylation with TPN (Equation 2) (5).

It was previously shown that the ATP requirement for reductive (photosynthetic) CO_2 assimilation by chloroplasts was satisfied only, if Reactions 1 and 2 were, in an appropriate balance, allowed to proceed simultaneously (1). The balance between these two reactions was maintained in this instance by including in the reaction mixture a minute amount of FMN (Table I) but similar results were also obtained in the presence of minute amounts of vitamin K_3 (1, 6; also see Fig. 2).

$$n\ ADP + n\ H_3PO_4 \xrightarrow[\text{chloroplasts}]{\text{light}} n\ ATP \qquad (1)$$

$$2\ TPN + 2\ H_2O + 2\ ADP + 2\ H_3PO_4 \xrightarrow[\text{chloroplasts}]{\text{light}} 2\ TPNH_2 + O_2 + 2\ ATP \qquad (2)$$

As shown in Table I, ammonia suppressed almost completely the formation of ATP in light, whether by Reaction 1 or 2. In the presence of ammonia, illuminated chloroplasts generated, apart from oxygen, only one component of assimilatory power, $TPNH_2$. Table II shows that under these conditions both the carboxylative and reductive phases of CO_2 assimilation were sharply curtailed. The inhibition of the carboxylative phase by ammonia was measured by the decrease in the total CO_2 fixed when ribose-5-P was supplied as the CO_2 acceptor. The lack of ATP blocked the conversion of ribose-5-P to ribulose-di-P which reacts with CO_2 in the carboxylation reaction to give 2 molecules of 3-PGA. (References are cited in the companion article (3).)

When ribulose-di-P was added as the CO_2 acceptor, the carboxylation reaction occurred, resulting in the cleavage of ribulose-di-P by CO_2 to two molecules of 3-PGA. Here the inhibitory effect of ammonia on total CO_2 fixation was only slight (Table II). But the shortage of ATP prevented the phosphorylation of 3-PGA to 1,3-diPGA and therefore blocked its reduction to triosephosphate by $TPNH_2$. $TPNH_2$, the photosynthetic "reducing power," continued to form in light, but without ATP it was powerless to reduce 3-PGA. Thus, in the presence of ribulose-

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[1] The abbreviations used are: PGA, 3-phosphoglyceric acid; 1,3-diPGA, 1,3-diphosphoglyceric acid; FMN, flavin mononucleotide.

TABLE I

Uncoupling of photosynthetic phosphorylation from TPN reduction by ammonia

Each vessel contained in a final volume of 3 ml: broken chloroplasts (C_{18}) containing 0.5 mg of chlorophyll; chlorophyll extract equivalent to 2 mg of chlorophyll; and the following in μmoles: Tris, pH 7.5, 80; $MgCl_2$, 5; $MnCl_2$, 2; sodium ascorbate, 10; TPN, 4; ADP, 10; $K_2HP^{32}O_4$, 10; FMN, 0.001 and NH_4Cl as indicated.

Concentration of NH_4Cl	$TPNH_2$ formed	P esterified	Inhibition of phosphorylation
	μmoles	*μmoles*	%
0	3.7	9.4	
5×10^{-4} M	3.6	7.7	18
10^{-3} M	3.6	5.0	47
10^{-2} M	3.4	0.6	94

di-P the inhibition of ATP formation by ammonia resulted in the accumulation of 3-PGA, indicating a curtailment of the reductive phase of CO_2 assimilation.

Ammonia inhibition of CO_2 fixation by chloroplasts has recently been reported by Gibbs and Calo (7). In their experiments with whole chloroplasts the inhibition by ammonia was not relieved by added ATP. In our experiments with reconstituted chloroplasts, the use of ATP in a prior conversion of ribose-5-P to ribulose-di-P was effective in virtually preventing the inhibition of the carboxylative phase of CO_2 assimilation by ammonia.

Effect of Dinitrophenol—Dinitrophenol inhibits photosynthetic phosphorylation but at concentrations (beginning at about 10^{-3} M) higher than those which uncouple oxidative phosphorylation (8, 9, 4). The effect of dinitrophenol on CO_2 assimilation was investigated with the use of, as in the case of ammonia, either ribose-5-P or ribulose-di-P as CO_2 acceptors. The results are given in Table III.

Dinitrophenol, at a concentration of 2×10^{-3} M, inhibited total CO_2 fixation 82% in the presence of ribose-5-P. The sole product of the diminished CO_2 fixation was PGA (Table III). These results indicate that both the carboxylative and reductive phases of CO_2 assimilation were strongly inhibited at a concentration of dinitrophenol, which is known to inhibit ATP formation in light.

Concordant evidence for dinitrophenol inhibition of the reductive phase of CO_2 assimilation was obtained when ribulose-di-P was supplied as the CO_2 acceptor. In this case total CO_2 fixation was inhibited only 26% (at 2×10^{-3} M dinitrophenol), but PGA accumulated as the predominant product of CO_2 fixation (Table III). The reduction of PGA to triosephosphate was impeded.

Competition for ATP by Hexokinase-glucose System—Another demonstration of the dependence of the carboxylative and reductive phases on photochemically generated ATP was obtained by the addition of a hexokinase-glucose system to the reaction mixture. In these experiments no inhibitor of photophosphorylation was used. ATP was allowed to form in light but its availability for CO_2 assimilation was reduced by the competition of the added hexokinase-glucose system. The results are summarized in Table IV.

The competition of the hexokinase-glucose system for ATP decreased total CO_2 fixation 80% in the presence of ribose-5-P. These results indicate that the carboxylative phase was limited

TABLE II

Effect of ammonia on CO_2 assimilation in light in presence of ribose-5-P or ribulose diphosphate

Each vessel contained in a final volume of 2.5 ml: broken chloroplasts (C_{18}) containing 0.5 mg of chlorophyll; chlorophyll extract equivalent to 2 mg of chlorophyll; and the following in μmoles: Tris buffer, pH 7.5, 80; $MgCl_2$, 5; $MnCl_2$, 2; sodium ascorbate, 10; TPN, 0.3; ADP, 0.5; sodium phosphate pH 7.5, 5; FMN, 0.001; $NaHC^{14}CO_3$, 10; and 1 μmole of either ribose-5-P or ribulose-di-P.

Concentration of NH_4Cl	Ribose-5-P		Ribulose-di-P		Total $C^{14}O_2$ fixed as	
	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed	Inhibition	Phosphoglycerate	Sugar phosphates*
	c.p.m.	%	*c.p.m.*	%	%	%
0	425,000		376,000		3	97
10^{-3} M	180,000	58	302,000	19	90	10
10^{-2} M	85,000	80	294,000	20	100	

* Sugar mono- and diphosphates and dihydroxyacetone phosphate.

TABLE III

Effect of dinitrophenol (DNP) on CO_2 assimilation in light in presence of ribose-5-P (I) and ribulose diphosphate (II)

Experimental conditions as in Table II.

Expt. No.	Concentration of DNP	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed as	
				Phosphoglycerate	Sugar phosphates
		c.p.m.	%	%	%
I	0	345,000		6	92
	10^{-4} M	331,000	3	8	90
	10^{-3} M	185,000	47	59	38
	2×10^{-3} M	62,000	82	100	
II	0	870,000		30	70
	10^{-4} M	810,000	6	25	75
	10^{-3} M	680,000	21	55	44
	2×10^{-3} M	630,000	26	80	20

TABLE IV

Effect of hexokinase system (HK) on CO_2 assimilation in light in presence of ribose 5-phosphate or ribulose diphosphate

Reaction mixture as in Table II, except that 5 μmoles of reduced glutathione were added. In the hexokinase treatment, 20 μmoles of glucose and 1.5 mg of a crude hexokinase preparation (Type II, Sigma Chemical Company) were added to the reaction vessel.

Treatment	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed as	
			Phosphoglycerate	Sugar phosphates
	c.p.m.	%	%	%
Ribose-5-P	410,000			
Ribose-5-P, HK	84,000	80		
Ribulose-di-P	815,000		26	73
Ribulose-di-P, HK	670,000	17	100	

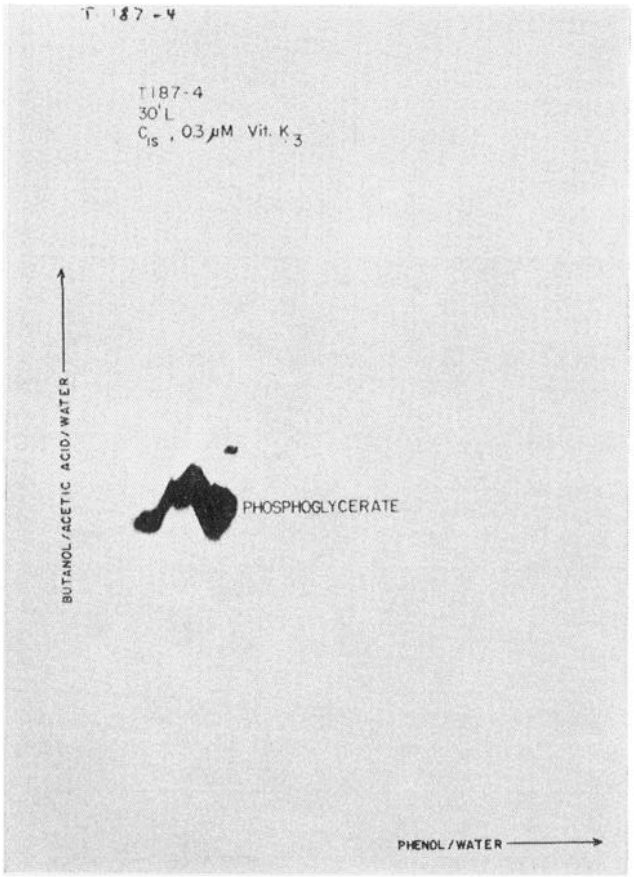

FIG. 1. Radioautograph of a chromatogram showing products of photosynthetic $C^{14}O_2$ assimilation by illuminated chloroplasts in the presence of 0.3 μmole of vitamin K_3. Reaction mixture as in Table II, except that FMN and pentose phosphates were omitted and 0.3 μmole of glucose-1-P was added.

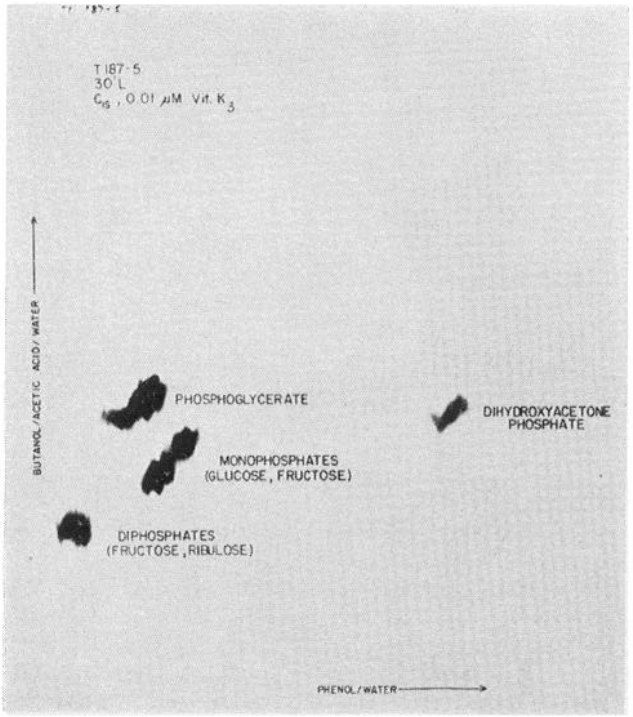

FIG. 2. Radioautograph of a chromatogram showing products of photosynthetic CO_2 assimilation by illuminated chloroplasts in the presence of 0.01 μmole of vitamin K_3. Reaction mixture as in Table II, except that FMN and pentose phosphates were omitted and 0.3 μmole of glucose-1-P was added.

by a shortage of ATP. Evidence for the curtailment of the reductive phase was found when ribulose-di-P was supplied as the CO_2 acceptor. Here total CO_2 fixation was inhibited only 17%, but no reduction of PGA occurred.

The competition for ATP, between an added hexokinase-glucose system and photosynthetic CO_2 assimilation, suggests that the feeding of glucose to intact, photosynthesizing cells may, by favoring the hexokinase reaction, bring about an apparent decrease in the rate of photosynthesis.

Inhibition of Reductive Phase of CO_2 Assimilation through Shortage of $TPNH_2$

Effect of Vitamin K_3—In the experiments described so far the reductive phase of CO_2 assimilation was inhibited by a shortage of ATP. The reductive phase was also inhibited, in the presence of abundant ATP, through a shortage of $TPNH_2$. Thus, a shortage of either ATP or $TPNH_2$ will prevent the reductive (photosynthetic) CO_2 assimilation.

The procedure used to demonstrate the dependence of the reductive phase on $TPNH_2$ was based on the previously reported effects of vitamin K, FMN, or phenazine methosulfate on noncyclic photophosphorylation (2, 6, 1). The addition of one of these substances, at a relatively high concentration, converts noncyclic photophosphorylation (Equation 2) to the cyclic type (Equation 1). Oxygen evolution and the accumulation of $TPNH_2$ are suppressed, phosphorylation is sharply increased, and the principal product of the light reaction is ATP (2, 6).

Under these conditions, ATP is abundant and the carboxylative phase of CO_2 assimilation should proceed without hindrance, resulting in the formation of PGA. But PGA would accumulate, since its reduction to triosephosphate would be blocked by lack of $TPNH_2$. Evidence that this occurred in the presence of 0.3 μmole of vitamin K_3 is shown in Fig. 1. At the lower concentration of vitamin K_3, when both cyclic and noncyclic photophosphorylations were possible, a normal reductive pattern of CO_2 assimilation in chloroplasts was observed (Fig. 2) (*cf.* 1).

Inhibitors of Individual Enzymes in CO_2 Assimilation

Effect of Arsenate and Phosphate—Weissbach *et al.* (10) reported that purified ribulose-di-P carboxylase was inhibited by arsenate and phosphate. It was therefore expected that arsenate and phosphate would strongly inhibit the carboxylative phase of CO_2 assimilation by the reconstituted chloroplast system. This expectation was not fulfilled. As shown in Table V, arsenate or phosphate, at concentrations high for inhibitors (11), was only mildly inhibitory to CO_2 fixation when ribulose-di-P was supplied as the CO_2 acceptor.

By contrast with the mild inhibition of the carboxylative phase, the addition of arsenate (but not of phosphate) strongly inhibited the reductive phase of CO_2 assimilation in chloroplasts. This is shown by the accumulation of PGA (Tables V and VI). When ribose-5-P was supplied as the CO_2 acceptor, arsenate was again only mildly inhibitory to total CO_2 fixation, a result which indicates no substantial inhibition of either the conversion of ribose-5-P to ribulose-di-P or of the carboxylation reaction. However,

TABLE V

Effect of phosphate and arsenate on CO_2 assimilation in light in presence of ribulose diphosphate

Experimental conditions as in Table II.

Treatment	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed as	
			Phosphoglycerate	Sugar phosphates
	c.p.m.	%	%	%
Control*	1,070,000		14	83
Arsenate				
10^{-3} M	910,000	14	58	41
5×10^{-3} M	880,000	17	87	12
10^{-2} M	790,000	26	100	0
Phosphate				
5×10^{-3} M	1,040,000			
10^{-2} M	1,100,000		17	81
3.7×10^{-2} M	750,000	30	18	80

* Contains 10^{-3} M phosphate.

TABLE VI

Effect of arsenate on CO_2 fixation in light in presence of phosphoglycerate or ribose 5-phosphate

Reaction mixture as in Table II. One μmole of PGA or of ribose-5-P was added, respectively.

Concentration of arsenate	Phosphoglycerate		Ribose-5-P			
	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed as	
					Phosphoglycerate	Sugar phosphates
	c.p.m.	%	*c.p.m.*	%	%	%
0	257,000		364,000		9	90
10^{-3} M	148,000	43	305,000	16	45	63
5×10^{-3} M	58,000	78	237,000	35	100	

in the presence of arsenate, CO_2 assimilation did not go beyond PGA, which accumulated as the main product (Table VI).

Further evidence for the inhibition by arsenate of PGA reduction was found when PGA was supplied as a precursor for the carboxylation phase (Table VI; also see Table V in ref. 3). Under these conditions arsenate strongly inhibited total CO_2 fixation. This suggests an insufficiency of the CO_2 acceptor, caused by the failure of the conversion of PGA to pentose phosphate, a series of reactions which includes the reduction of PGA to triosephosphate.

Arsenate thus seemed to be a strong inhibitor not of the carboxylative but of the reductive phase of CO_2 assimilation, which involves the reduction of PGA to glyceraldehyde-3-P. This effect of arsenate in hindering the reduction of PGA would be expected from the well known effect of arsenate in promoting the irreversible oxidation of glyceraldehyde-3-P to PGA as catalyzed either by the classical DPN-dependent triosephosphate dehydrogenase (12) or by the TPN-dependent enzyme from photosynthetic tissues (13, 14).

Arsenate and phosphate seem to be more effective inhibitors of CO_2 assimilation in whole chloroplasts (7) than in broken chloroplasts (15), similar to those used in the present investigation. An explanation of the inhibitory effect of phosphate on CO_2 fixation by whole chloroplasts was sought earlier (16) in a possible competition between photosynthetic phosphorylation and CO_2 assimilation. However, the work with reconstituted chloroplast systems (1, 3) now leaves little doubt that photosynthetic phosphorylation is a prerequisite for CO_2 assimilation in photosynthesis. Phosphate, within a physiological range of concentration, does not inhibit CO_2 assimilation.

Avron and Jagendorf (17) concluded that arsenate is a competitive inhibitor of photosynthetic phosphorylation, but they also observed little inhibition of ATP formation in light (Table IV in ref. 4) at the highest concentration of arsenate, 1.0×10^{-2} M, used in this investigation. This is in accord with the present findings that arsenate was only mildly inhibitory in the carboxylative phase, in which an ATP shortage would, in the presence of ribose-5-P, be reflected in a sharp curtailment of the total CO_2 fixed.

Effect of Iodoacetamide—In previous experiments with whole chloroplasts, iodoacetamide was found to be a strong inhibitor of CO_2 fixation but not of photosynthetic phosphorylation (8). With reconstituted chloroplasts iodoacetamide strongly inhibited the carboxylative phase, but only mildly the reductive phase of CO_2 assimilation (Table VII).

The strong inhibition of the carboxylative phase, as shown in the ribose-5-P series (Table VII), cannot be attributed to a shortage of ATP as, for example, in the case of ammonia inhibition (Table II), since on present evidence, iodoacetamide, at the concentration used, does not inhibit photophosphorylation. The

TABLE VII

Effect of iodoacetamide (IAA) on CO_2 assimilation in light in presence of ribose 5-phosphate or ribulose diphosphate

Experimental conditions as in Table II.

Concentration of IAA	Ribose-5-P		Ribulose-di-P			
	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed	Inhibition	Total $C^{14}O_2$ fixed as	
					Phosphoglycerate	Sugar phosphates
	c.p.m.	%	*c.p.m.*	%	%	%
0	438,000		690,000		17	81
10^{-4} M	380,000	13	645,000	6	17	80
10^{-3} M	130,000	70	530,000	24	12	87
10^{-2} M	18,000	96	440,000	36	48	49

TABLE VIII

Effect of cyanide on CO_2 fixation in light in presence of ribulose diphosphate

Experimental conditions as in Table II.

Concentration of cyanide	Total $C^{14}O_2$ fixed	Inhibition
	c.p.m.	%
0	632,000	
10^{-4} M	238,000	62
2×10^{-4} M	114,000	82
10^{-3} M	31,000	95

inhibitory effect of iodoacetamide on the total CO_2 fixed in the presence of ribose-5-P is explained by its inhibition of the conversion of ribose-5-P to ribulose-di-P, and in this manner reducing the supply of ribulose-di-P for the carboxylation reaction. Phosphoribulokinase, needed for the conversion of ribulose-5-P to ribulose-di-P was found to be a sulfhydryl enzyme (18), and its inhibition by iodoacetamide might be expected. But it is not excluded that iodoacetamide may also inhibit pentose isomerase which catalyzes the conversion of ribose-5-P to ribulose-5-P.

The mild inhibition by iodoacetamide of the reductive phase of CO_2 assimilation, as shown by ribulose-di-P series in Table VII, indicates that, under our experimental conditions, there was no strong inhibition of the phosphoglyceric acid kinase or of the TPN-linked triosephosphate dehydrogenase by this inhibitor. It was previously reported (13) that the TPN-linked triosephosphate dehydrogenase is more resistant to iodoacetamide inhibition than the DPN-linked enzyme.

Iodoacetamide inhibition of CO_2 fixation by whole and broken chloroplasts has recently been reported by Gibbs and Calo (7, 15) who observed that broken chloroplasts are more sensitive to the action of this inhibitor than the intact preparations.

Effect of Cyanide—Cyanide inhibition of photosynthesis has been the subject of many investigations, ever since it was first discovered by Warburg (19). Cyanide was previously found to inhibit the dark CO_2 assimilation in isolated chloroplasts (20). It was therefore of special interest to find that cyanide was a strong, and in our experience so far, the only effective inhibitor of ribulose-di-P carboxylase. As shown in Table VIII, in the presence of ribulose-di-P as the CO_2 acceptor, total CO_2 fixation was markedly reduced by low concentrations of cyanide.

SUMMARY

The dependence of the dark reactions of photosynthesis, concerned with CO_2 assimilation, on two products of the light reactions, reduced triphosphopyridine nucleotide ($TPNH_2$) and adenosine triphosphate (ATP), and the site of action of these two compounds in a reconstituted chloroplast system, were investigated under different experimental conditions.

The inhibition by ammonia or dinitrophenol of ATP formation in light showed two sites of ATP action and resulted in the inhibition of two phases of CO_2 assimilation: the carboxylative phase which includes the phosphorylation of pentose monophosphate and the fixation of CO_2, and the reductive phase, which includes the reduction of phosphoglyceric acid and the formation of hexose phosphate.

The carboxylative and reductive phases of CO_2 assimilation were also inhibited when ATP was allowed to form in light, but its availability for CO_2 assimilation was diminished by the competition of an added hexokinase-glucose system.

The requirement for $TPNH_2$ in the reductive phase of CO_2 assimilation was shown under conditions when photophosphorylation occurred but $TPNH_2$ formation was suppressed in light by the addition of appropriate amounts of vitamin K_3.

CO_2 assimilation was inhibited by certain inhibitors under conditions when the supply of neither ATP nor $TPNH_2$ was limiting. Cyanide was found to inhibit strongly the carboxylation of ribulose diphosphate; arsenate inhibited the reduction of phosphoglyceric acid; and iodoacetamide inhibited the conversion of ribose 5-phosphate to ribulose diphosphate.

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