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Photosynthetic Enhancement Studied in Intact Spinach Chloroplasts[1]

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ABSTRACT

The Emerson enhancement effect was evaluated in the intact spinach (*Spinacia oleracea* var. Long Standing Bloomsdale) chloroplast by monitoring the uptake of $^{14}CO_2$ during illumination by 640 nm and 720 nm lights. Low levels (about 10 μM) of fructose 1,6-diphosphate, ribose 5-phosphate, and glycerate 3-phosphate stimulated the rate of photosynthesis and abolished enhancement values observed in their absence. Concentrations of the two sugar phosphates at levels of 1 mM responded similarly. In contrast, 1 mM glycerate 3-phosphate inhibited the rate of photosynthesis and increased enhancement. The exchange of glycerate 3-phosphate for glyceraldehyde 3-phosphate was speculated to be a factor underlying the decrease in photosynthesis and the increase in enhancement. Glucose 6-phosphate, NADPH, and L-malate did not influence photosynthesis or enhancement.

The uncoupler, *p*-trifluoromethoxyphenylhydrazone, decreased the rate of photosynthesis but did not change the enhancement values. ATP (0.2 to 1 mM) had an occasional stimulating effect on CO_2 fixation but no effect on enhancement. Magnesium ions inhibited photosynthesis and decreased the enhancement values. It was concluded that the enhancement phenomenon reflects events of the photosynthetic carbon reduction cycle as well as the photochemical act.

Attempts to evaluate the Emerson enhancement effect in isolated chloroplasts have been numerous in recent years (11), but surprisingly all reports but two have measured enhancement solely in partial reactions of the chloroplast. In these contributions (9, 10), enhancement was observed for O_2 evolution accompanying CO_2 fixation by intact chloroplasts. In the present investigation, uptake of $^{14}CO_2$ by the intact spinach chloroplast was followed to elucidate the Emerson enhancement effect. Conditions necessary for the demonstration of this effect in the intact organelle are reported. In addition, the regulation of enhancement by intermediates of the photosynthetic carbon reduction cycle and other substances has been evaluated. This study has shown that, as viewed by CO_2 fixation, regulation of enhancement involves both the photochemistry and the "dark steps" of photosynthesis.

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MATERIALS AND METHODS

Spinach (*Spinacia oleracea* var. Long Standing Bloomsdale) was grown in a controlled environmental chamber at 25 C during 8 hr of light (2400 ft-c produced by white fluorescent and tungsten lamps) and at 20 C during 16 hr of dark. The spinach, obtained from Agway, Inc., was grown in vermiculite and was maintained on Hoagland's solution upon germination. Fully expanded leaves were collected about 6 to 8 weeks after germination.

Intact chloroplasts were isolated by the method of Jensen and Bassham as modified by Gibbs and Robinson (5). The chloroplasts containing 100 to 200 mg Chl/ml were stored on ice in a tube wrapped in aluminum foil during the experiments. Chl was determined by the procedure of Arnon (1). Electron micrographs showed that this procedure produced 60 to 80% intact chloroplasts.

Monochromatic light was produced by passing white light through interference filters purchased from Baird Atomic, Inc. The filters (5 × 5 cm) were blocked to infinity, peaked at 640 nm and 720 nm with a half-width of 20 nm. The light source for the 640 nm filter was produced by an Airequipt/avventura 380 projector which contained a Sylvania 500-w lamp and a 10-cm f2.8 luminan lens system. A General Electric 1000-w projector lamp provided the light for the 720 nm filter. Metal screens of varying mesh acted as neutral density filters to adjust the intensity of the light. An Eppley thermopile was employed to determine the light energy generated. The light of 720 nm had an intensity of 1.27×10^4 ergs sec^{-1} cm^{-2}. The light intensity of the 640 nm beam was 0.16×10^4 ergs sec^{-1} cm^{-2}.

A standard reaction mixture contained in a final volume of 2 ml: 50 mM HEPES-NaOH, pH 7.8; 0.33 M sorbitol; 2 mM $NaNO_3$; 20 mM NaCl; 2 mM sodium ascorbate; 1 mM manganese chloride; 1 mM magnesium chloride; 5 mM $Na_4P_2O_7$; 2 mM Na_2EDTA, chloroplasts containing 40 to 100 μg Chl and 5 mM $NaH^{14}CO_3$ with a specific radioactivity of 10 to 15 μCi/μmole.

Chloroplasts were added in darkness to the two cuvettes containing the standard reaction mixture. The mixture was equilibrated for 3 min in darkness with frequent stirring. The cuvettes were maintained at a temperature between 24 and 28 C using a series of water baths to filter the light beams. A zero time aliquot was removed, and then the light was switched on. The cuvettes were illuminated on both sides. Aliquots were removed at intervals, and the reaction was stopped with 25 μl of concentrated formic acid. Duplicate aliquots of 0.05 ml for each time point were placed on aluminum planchets, dried, and then counted on a low background gas flow counter (Nuclear Chicago).

To ensure reproducibility, various parameters were tested and standardized. A control was run at the beginning and

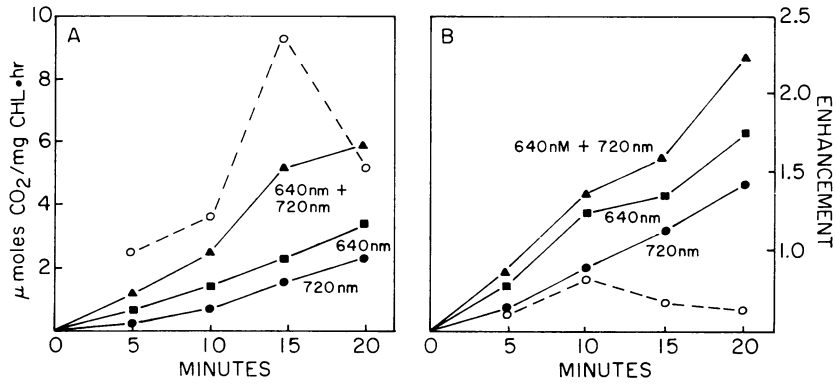

FIG. 1: Effect of fructose-1,6-diP on enhancement. The experiments were performed in the standard reaction mixture. The results were obtained from experiments run simultaneously with identical chloroplast preparations. Radioactivity fixed is given in terms of an aliquot of 0.05 ml. A: control; B: 1 mM fructose-1,6-diP. The dashed line represents the enhancement values.

the end of each experiment to determine if there was loss in the photosynthetic capacity of the plastids. Chloroplasts stored in a glass tube in the dark on ice did not deteriorate more than 10% with respect to $^{14}CO_2$ photoassimilation in white light over a 3-hr period. The relative order of illumination with the 640 nm, 720 nm, or both lights was investigated and no significant differences were detected. A series of experiments with chloroplasts was performed to obtain the maximum enhancement values by varying the intensities of the 640 nm and 720 nm beams. A ratio of 8 to 1 for the intensities of the 720 nm and 640 nm beams, respectively, proved optimum. The rate of $^{14}CO_2$ fixation increased linearly with respect to the 640 nm and 720 nm beams. This relationship provided reasonable assurance that enhancement in our preparations was dependent on the wavelength and was not the result of nonlinear effects. The combined intensities of these two lights were 10% saturating in relation to photosynthesis in white light. All results represent an average of two sets of data which never differed by more than 5%. Each experiment was carried out at least twice and in each case with a different preparation of chloroplasts. Enhancement is expressed in this paper by the following formulation:

$$\text{Enhancement} = \frac{\text{rate (640 nm + 720 nm)} - \text{rate (640 nm)}}{\text{rate (720 nm)}}$$

The enhancement apparatus and conditions were tested by monitoring $^{14}CO_2$ uptake in the green alga, *Volvox cateri*, for a total of 15 min at three 5-min intervals. The enhancement values were 2 at 5 min, 2.57 at 10 min, and 2.75 at 15 min.

Fructose-1,6-diP, ribose-5-P, glycerate-3-P, L-ascorbate, L-malate, ATP, and NADPH were purchased from Sigma Chemical Co. *p*-Trifluoromethoxyphenylhydrazone and DCMU were kindly provided by P. G. Heytler. $Ba^{14}CO_3$ was obtained from New England Nuclear.

RESULTS AND DISCUSSION

Photosynthetic Intermediates. A representative experiment is illustrated in Figure 1A. A slight lag in $^{14}CO_2$ assimilation noted in a number of reports where photosynthesis was driven by white light was also seen in monochromatic light even at our low light intensities. Generally, enhancement was lowest during the initial 5-min interval. The enhancement value rose between 5 and 15 min and then decreased in the 15- to 20-min interval. The lag in CO_2 assimilation in chloroplasts has been shown by a number of investigators to be reduced or eliminated by some intermediates of the photosynthetic carbon reduction

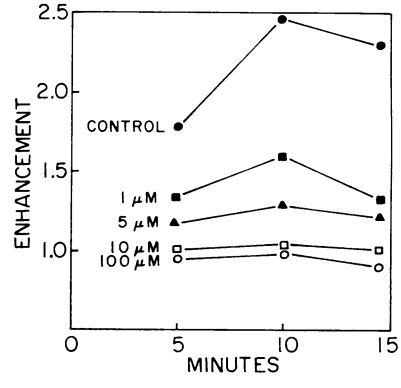

FIG. 2. Effect of fructose-1,6-diP concentration on enhancement.

cycle (4). In this study, we confirmed that glycerate-3-P, fructose-1,6-diP, or ribose-5-P reduced the lag phase and stimulated photosynthesis. Fructose-1,6-diP at 1 mM (Fig. 1B) or ribose-5-P (data not shown) abolished the Emerson enhancement effect. Subsequent experiments demonstrated that at 10 μM fructose-1,6-diP, photosynthesis reached a maximum rate with a concomitant elimination of enhancement (Fig. 2). A similar result was observed with ribose-5-P (data not shown). It may well be that the decrease in the enhancement values observed in the 15- to 20-min interval (Fig. 1A) was the result of an accumulation of intermediates of the photosynthetic carbon cycle in the plastids and in the suspending medium.

Of the intermediates of the carbon cycle which are known to penetrate the chloroplast, only glycerate-3-P has been reported to show a biphasic effect with respect to CO_2 fixation (Andersen and Gibbs, unpublished observations). In our experiments, this phosphorylated acid usually stimulated CO_2 fixation at concentrations up to approximately 0.5 mM but was inhibitory at higher levels. In the individual or combined beams, 10 μM glycerate-3-P stimulated, while 1 mM inhibited, CO_2 fixation (Table I). Thus, glycerate-3-P behaved similarly to the sugar phosphates only at the lower level. The response of enhancement to glycerate-3-P differed somewhat from that of the sugar phosphates. The lower concentrations tended to diminish the enhancement values, but at the inhibitory concentrations of glycerate-3-P, the values increased strikingly. The exchange of glycerate-3-P for glyceraldehyde-3-P may be the underlying cause of the higher enhancement values. Thus, following the lead of Heldt *et al.* (8), Andersen and Gibbs

Table I. *Effect of Glycerate-3-P Levels on Enhancement*

Glycerate-3-P Concn	Time of Illumination	Rate of Photosynthesis in			Enhancement
		720 nm	640 nm	720 nm and 640 nm	
μM	min	μmoles/mg Chl·hr			
0	5	0.92	1.91	3.25	1.46
	10	1.23	2.36	4.56	1.79
	15	1.37	2.84	5.36	1.89
1	5	1.02	1.67	3.18	1.48
	10	1.35	2.34	4.25	1.41
	15	1.53	2.78	5.14	1.54
10	5	1.56	1.94	4.63	1.72
	10	1.86	2.87	5.42	1.37
	15	2.06	3.52	5.99	1.20
100	5	1.02	1.72	4.09	2.32
	10	1.29	2.09	4.56	1.91
	15	1.46	2.45	4.95	1.71
1000	5	0.18	0.30	0.89	3.28
	10	0.19	0.44	1.22	4.11
	15	0.21	0.55	1.52	4.62

(unpublished observations) interpreted their data on glycerate-3-P inhibition of CO_2 fixation but not of O_2 evolution as the result of the exchange mechanism. Clearly, the rate of exchange would depend upon the concentration of the two intermediates of the carbon cycle. Since the concentration of glyceraldehyde-3-P was related to the rate of CO_2 assimilation, the strongest inhibition should be and was observed in the individual light beams and with 1 mM glycerate-3-P. We, therefore, propose that a stress on the photosynthetic carbon reduction cycle resulting in a decreased regeneration of ribulose-1,5-diP had a strong influence on the enhancement effect.

Glucose-6-P at 1 mM had no effect on CO_2 fixation or enhancement (data not shown). This result was expected, since the chloroplast membrane was not permeable to this sugar phosphate (4).

Effect of Other Substances. The ability of ATP to penetrate the whole chloroplast in a medium of 0.35 M NaCl or 0.33 M sorbitol has been clouded with conflicting reports (16). Heldt (8) has reported a maximum rate of 5 μmoles ATP/mg Chl·hr. With respect to enhancement, McSwain and Arnon (10) have reported that the addition of 0.2 mM ATP abolished the photosynthetic enhancement of O_2 evolution accompanying CO_2 assimilation by intact chloroplasts. Concentrations of 0.2 to 1 mM ATP stimulated CO_2 fixation 20 to 100% in some of our preparations. This effect could not be demonstrated repeatedly and may well have been the result of a varying mixture of broken and whole plastids. Enhancement was not altered under conditions where ATP was effective or ineffective with respect to CO_2 fixation.

Similar results were observed when O_2 evolution was monitored in these preparations. For example, in an experiment where 0.2 mM ATP and 1 mM ATP stimulated O_2 evolution from a control value in the combined beams of 7.8 μmoles/mg Chl·hr to 8.4 and 8.6, respectively, the enhancement values were 2.08 (control), 2.10, and 2.12.

Since the results obtained with ATP could be considered equivocal due to lack of entry into the chloroplast, the effect of an uncoupler on enhancement was determined. *p*-Trifluoromethoxyphenylhydrazone at a concentration of 0.025 μM inhibited CO_2 fixation in the individual and combined beams by roughly 50% with no effect on enhancement (Table II). In parallel experiments where O_2 evolution was determined and the concentration of the uncoupler was 0.025 μM and 0.05 μM, the photosynthetic rate was impaired 49% and 63%, respectively, but enhancement was once again unaffected.

Prompted by the reports of Sun and Sauer (14), Sinclair (13), and McSwain and Arnon (10) that Mg^{2+} could influence the enhancement of NADP in broken chloroplast reactions, we tested its effect on CO_2 fixation in our intact preparations (Table III). As reported by Avron and Gibbs (2), Mg^{2+} were inhibitory to CO_2 fixation and, in turn, decreased the enhancement values and, indeed, below unity at the highest concentration of Mg^{2+} tested. Clearly, the intact organelle was not a suitable preparation to demonstrate a role for Mg^{2+} in photosynthetic reactions housed within the envelope (6).

Finally, we observed that 1 mM NADPH and 1 mM L-malate did not affect either the rate of $^{14}CO_2$ assimilation or enhancement. While the pyridine nucleotide is reported not to penetrate the intact chloroplast, the dicarboxylic acid is known to serve as a shuttle substrate (7).

Table II. *Effect of F_3CCP on Enhancement*

The experiment was performed in the standard mixture. The control and the experiment with 0.025 μM F_3CCP were run with the same chloroplast preparation.

Time of Illumination	Control				F_3CCP			
	Rate of photosynthesis in				Rate of photosynthesis in			
	720 nm	640 nm	720 nm and 640 nm	Enhancement	720 nm	640 nm	720 nm and 640 nm	Enhancement
min	μmoles/mg Chl·hr				μmoles/mg Chl·hr			
5	0.6	0.8	2.0	2.00	0.3	0.4	1.1	2.33
10	2.2	2.6	6.8	1.91	1.1	1.2	4.0	2.55
15	3.8	4.7	10.6	1.55	1.9	2.4	5.4	1.58

Table III. *Effect of Mg^{2+} on Enhancement*

Mg^{2+} Concn	Time of Illumination	Rate of Photosynthesis in			Enhancement
		720 nm	640 nm	720 nm and 640 nm	
μM	min	μmoles/mg Chl·hr			
0	5	2.9	3.4	7.8	1.52
	10	6.4	8.7	20.7	1.88
	15	10.3	15.0	33.7	1.82
1	5	1.1	1.1	2.9	1.10
	10	2.6	2.7	6.5	1.46
	15	3.9	4.7	9.0	1.64
2	5	1.1	1.0	2.0	0.91
	10	1.9	2.1	4.5	1.26
	15	2.8	3.1	6.3	1.14
4	5	0.7	0.9	1.6	0.78
	10	1.5	1.8	3.4	1.07
	15	2.3	2.7	4.5	1.00
8	5	0.9	0.8	1.4	0.61
	10	1.5	1.4	2.6	0.80
	15	1.8	1.9	3.0	0.67

CONCLUSION

These experiments confirm and extend earlier reports (9, 10) that the Emerson enhancement effect and its regulation can be studied in the intact chloroplast actively engaged in CO_2 fixation. The isolated organelle has some advantages with respect to the mother cell. First, the membrane of the plastid is permeable to substances not easily evaluated in the whole cell; second, the determination of enhancement is not obscured by the biochemistry of respiration; and third, the interaction between the photochemistry and the photosynthetic carbon reduction cycle which allows enhancement to be expressed can be monitored.

That fructose-1,6-diP, ribose-5-P, and glycerate-3-P eliminated the lag and stimulated CO_2 fixation in the plastid has been repeatedly demonstrated (4). Furthermore, in whole chloroplasts assimilating CO_2, these carbon cycle intermediates at roughly 10 μM have also been reported to overcome the Warburg effect (12), uncouplers of photosynthetic electron transport, and inhibitors of the photosynthetic carbon reduction cycle (4). To explain the elimination of the lag, it has been envisaged that the cycle intermediates are able to penetrate the chloroplast, prime the Calvin cycle, and fill the pools during the inductive phase of photosynthesis. A similar reasoning can be applied here. If a high ratio between glycerate-3-P and glyceraldehyde-3-P (or ribulose-1,5-diP) results in higher enhancement values, then elevating the level of ribulose-1,5-diP by intermediates of the carbon cycle would, in turn, lower the values. The recent demonstrations (3, 15) that these intermediates can affect CO_2 fixation by regulating ribulose-1,5-diP carboxylase may also be a critical factor. The relief of apparently divergent inhibitory effects such as O_2, uncouplers, and inhibitors by the carbon cycle intermediates has not been satisfactorily explained. Clearly, it is also difficult to assign a definitive mechanism to these intermediates in regulating enhancement.

Finally, the enhancement phenomenon is currently envisaged to reflect events solely in terms of the photochemical act. We question this interpretation and propose on the basis of the glycerate-3-P (Table I) and uncoupler (Table II) data that reducing power (reduced pyridine nucleotide and ATP) is not necessarily causally related to the enhancement effects evaluated in the isolated spinach preparations. We would broaden the current concept of the enhancement phenomenon to include the photosynthetic carbon reduction cycle as well as events in the photochemistry.

LITERATURE CITED

1. Arnon, D. I. 1949. Copper enzymes in chloroplasts. Polyphenoloxidases in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Avron, M. and M. Gibbs. 1974. Properties of phosphoribulokinase of whole chloroplasts. Plant Physiol. 53: 136–139.
3. Buchanan, B. B. and P. Schurmann. 1972. A regulatory mechanism for CO_2 assimilation in plant photosynthesis: activation of ribulose-1,5-diphosphate carboxylase by fructose-6-phosphate and deactivation by fructose-1,6-diphosphate. FEBS Lett. 23: 157–159.
4. Gibbs, M. 1971. Carbohydrate metabolism by chloroplasts. *In:* M. Gibbs, ed., Structure and Function of Chloroplasts. Springer-Verlag, Berlin.
5. Gibbs, M. and J. M. Robinson. 1974. Photosynthetic CO_2 incorporation in isolated spinach chloroplasts. *In:* A San Pietro, ed., Experimental Plant Physiology. C. V. Mosby, St. Louis. pp. 13–20.
6. Gimmler, H., G. Schafer, and U. Heber. 1974. Low permeability of the chloroplast envelope towards cations. *In:* Third International Congress of Photosynthesis, Rehovoth, Israel. p. 37.
7. Heldt, H. W. and L. Rapley. 1970. Unspecific permeation and specific uptake of substances in spinach chloroplasts. FEBS Lett. 7: 139–147.
8. Heldt, H. W., E. Sauer, and L. Rapley. 1971. Differentiation of the permeability properties of the two membranes of the chloroplast envelope. *In:* G. Forti and M. Avron, eds., Proceedings of the IInd International Congress on Photosynthesis, Stresa, Vol. II. pp. 1345–1355.
9. McSwain, B. D. and D. I. Arnon. 1968. Enhancement effects and the identity of the two photochemical reactions of photosynthesis. Proc. Nat. Acad. Sci. U.S.A. 61: 989–996.
10. McSwain, B. D. and D. I. Arnon. 1972. Factors influencing photosynthetic enhancement in isolated chloroplasts. Biochem. Biophys. Res. Commun. 49: 68–75.
11. Myers, J. 1971. Enhancement studies in photosynthesis. Annu. Rev. Plant Physiol. 22: 289–312.
12. Robinson, J. M. and M. Gibbs. 1974. Photosynthetic intermediates, the Warburg effect, and glycolate synthesis in isolated spinach chloroplasts. Plant Physiol: 53: 790–797.
13. Sinclair, J. 1972. The reversible abolition of enhancement. Plant Physiol. 50: 778–783.
14. Sun, A. S. and K. Sauer. 1972. Pigment systems and electron transport in chloroplasts. II. Emerson enhancement in broken spinach chloroplasts. Biochim. Biophys. Acta 256: 409–427.
15. Walker, D. A. 1973. Photosynthetic induction phenomena and the light activation of ribulose-1,5-diphosphate carboxylase. New Phytol. 72: 209–235.
16. Walker, D. A. 1974. Chloroplast and cell; concerning the movement of certain key metabolites etc. across the chloroplast envelope. *In:* D. H. Northcote, ed., Med. Tech. Publ. Int. Rev. Sci. Biochem. Ser. I, Vol. II. Butterworth, London. pp. 1–49.