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Influence of Inorganic Phosphate on Photosynthesis of Wheat Chloroplasts

II. RIBULOSE BISPHOSPHATE CARBOXYLASE ACTIVITY

F. MÄCHLER AND J. NÖSBERGER

Institut für Pflanzenbau, ETH-Zentrum, Universitätstrasse 2, CH-8092 Zürich, Switzerland

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ABSTRACT

Isolated wheat chloroplasts were pre-incubated in the dark in the presence of various concentrations of inorganic phosphate with or without carbon dioxide, oxaloacetate, glycerate, and 3-phosphoglycerate. The effect of subsequent illumination on photosynthetic oxygen evolution, ribulose bisphosphate carboxylase activity, ATP content, and ribulose bisphosphate content was investigated. Inorganic phosphate had little effect on ribulose bisphosphate carboxylase activity in darkness or during the initial phase of illumination, but it prevented the decline in activity that occurred during later stages of illumination, when photoreduction of CO_2 was decreasing in rate. Addition of inorganic phosphate to chloroplasts illuminated without phosphate restored the ribulose bisphosphate carboxylase activity, increased the ATP, and decreased the ribulose bisphosphate in the organelles. The responses to CO_2, oxaloacetate, glycerate, and 3-phosphoglycerate suggest that the decreased activity of ribulose bisphosphate carboxylase during photosynthesis results from ATP consumption.

Purified ribulose bisphosphate carboxylase was activated by inorganic phosphate, but this activation did not occur in the presence of ATP. ATP inhibited ribulose bisphosphate carboxylase when it was present in combination with various photosynthetic metabolites.

Inactivation of ribulose bisphosphate carboxylase in chloroplasts, illuminated in the absence of inorganic phosphate, is not due to lack of activation by inorganic phosphate or ATP. It may result from decreased stromal pH.

Key words: Ribulose bisphosphate carboxylase; Chloroplasts; Wheat; Phosphate; ATP.

INTRODUCTION

Ribulose bisphosphate carboxylase (RuBPCO) activity in chloroplasts is low in the dark and increases upon illumination (Sicher, 1982). Light activation may be attributed to an increase in stromal pH and Mg^{++} concentration (Werdan, Heldt, and Milovancev, 1975; Lorimer, Badger, and Andrews, 1976). Activity of RuBPCO in chloroplasts was high upon illumination for 8–10 min and then declined in a similar way to the rate of CO_2 fixation, suggesting that the decline in CO_2 fixation may have been caused by deactivation of the enzyme. However, the addition of 3-phosphoglycerate or glycerate doubled the initial rate of CO_2 fixation without increasing the RuBPCO activity (Stumpf and Jensen, 1982). The authors speculate that the

Abbreviations: ATP: adenosine triphosphate; P_i: inorganic phosphate; RuBP: ribulose bisphosphate; RuBPCO: ribulose bisphosphate carboxylase.

lack of correlation in the observed rates of photosynthetic CO_2 fixation and the initial activity of the RuBPCO is due to a discrepancy between measured and actual RuBPCO activity.

The present study investigates the cause for the decline of RuBPCO activity during photosynthesis. It may be the result of the consumption of PO_4^{3-} (P_i), since this is an activator of RuBPCO (Bhagwat, 1981). Furthermore, Heldt, Chon, and Lorimer (1978) suggest that P_i is a prerequisite for the light activation of RuBPCO in chloroplasts. The present study investigates four problems. (1) RuBPCO activity in illuminated chloroplasts, with and without P_i in the medium, is compared. (2) The influence of the P_i concentration in the medium on the contents of ATP and ribulose bisphosphate (RuBP) in the chloroplasts is investigated. (3) RuBPCO activity during the photoreduction of various substrates differing in their consumption of ATP and P_i is investigated (4). The inconsistency between the response of purified RuBPCO to P_i and of RuBPCO in chloroplasts to P_i is studied by measuring the activity of purified RuBPCO in the presence of ATP, P_i, and other metabolites.

MATERIALS AND METHODS

Chloroplast experiments

Plants were grown, chloroplasts were prepared and photosynthesis was measured as described earlier (Mächler, Schnyder, and Nösberger, 1984). Experiments were conducted in an oxygen electrode at a photon flux density of 300 μmol m^{-2} s^{-1}. Temperature was kept at 5 °C or 25 °C using a circulating water bath. To measure RuBPCO activity, 50 mm^3 samples of chloroplast suspensions were mixed rapidly into 450 mm^3 of a hypotonic medium containing detergent, RuBP and $NaH^{14}CO_3$ as described by Robinson, McNeil, and Walker (1979). After 1 min incubation at 20 °C, 100 mm^3 2 N HCl was added. ^{14}C, incorporated into acid stable compounds, was determined. ATP in extracts was measured by the luciferase method using the luminometer and assay chemicals from LKB Wallac (Turku, Finland). RuBP was measured in the same extracts by the method of Latzko and Gibbs (1974).

Purification of RuBPCO

The first leaves of 10 d old wheat seedlings (50 g) were homogenized in 100 cm^3 of 20 mM Tris HCl (pH 8·0), 10 mM mercaptoethanol, 1·0 mM EDTA, and $(NH_4)_2SO_4$ giving 37% saturation in the homogenate. The homogenate was filtered through cheese cloth and the filtrate centrifuged at 20 000 × *g* for 20 min. $(NH_4)_2SO_4$ was added to the supernatant to give 50% saturation. After 10 min centrifugation at 10 000 × *g* the supernatant liquid was decanted and the precipitate dissolved in extraction buffer (6·0 cm^3). The solution was applied to a chromatography column (25 mm × 500 mm) of Sephadex G-25 (course), equilibrated with extraction buffer. The eluate was applied to a column (25 mm × 1000 mm) of Sepharose Cl-6B, equilibrated with 5·0 mM Hepes KOH (pH 8·0), and 1·0 mM dithiothreitol. Eluted fractions containing high RuBPCO activity were combined and freeze-dried.

Experiments with purified RuBPCO

A sample of freeze-dried enzyme was dissolved in distilled water and the solution mixed with an equal volume of 200 mM Tris HCl (pH 8·6) containing 40 mM $MgCl_2$ and 20 mM $NaHCO_3$ and activated by incubation at 40 °C for 20 min. Activated enzyme was diluted 1:10 with 100 mM Tris HCl (pH 8·0) and 5·0 mM dithiothreitol giving concentrations of 1·0 mM $NaHCO_3$ and 2·0 mM $MgCl_2$. Effectors were added as indicated in Table 1 and solutions incubated for 20 min at 20 °C. 50 mm^3 samples were tested for RuBPCO activity in 0·5 cm^3 assay medium at 20 °C as described by Lorimer, Badger, and Andrews (1977).

RESULTS

1. RuBPCO activity in chloroplasts with and without P_i in the medium

Chloroplasts were pre-incubated in the dark at 20 °C with 5·0 mM P_i or without P_i. After 20 min, the suspensions were illuminated and samples taken at intervals for RuBPCO activity tests (Fig. 1), RuBPCO activity increased for 4 min. This increase was relatively independent of the P_i concentration in the medium. Thereafter, RuBPCO activity decreased considerably if the medium was deficient in P_i, but only slightly in the presence of 5·0 mM P_i. O_2 evolution was very low in both treatments. The results suggest that P_i does not affect RuBPCO activity

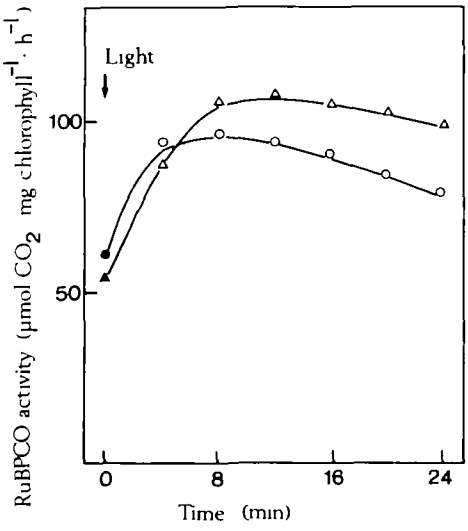

FIG. 1. RuBPCO activity in chloroplasts with and without P_i in the medium. Chloroplasts (28 μg chlorophyll cm^{-3} suspension) were pre-incubated in the dark at 20 °C in 50 mM Hepes KOH (pH 7·6), 0·4 M sorbitol, 1·0 mM $NaHCO_3$, 1·0 mM EDTA and 200 units catalase cm^{-3} suspension with either 5·0 mM P_i (△) or without P_i (○). After 20 min pre-incubation, chloroplasts were illuminated and samples taken at intervals for measurement of RuBPCO activity. Closed symbols represent measurements in the dark, before illumination.

in chloroplasts in the dark and during induction of photosynthesis after illumination. However, P_i seems to prevent RuBPCO from inactivation when photosynthesis proceeds.

2. The ATP and RuBP contents of illuminated chloroplasts as influenced by P_i and the relationship to RuBPCO activity

Chloroplasts were pre-incubated for 20 min in the dark without P_i and then illuminated (Fig. 2). After a few minutes of photosynthesis 0·0, 0·3, or 5·0 mM P_i was added. Oxygen evolution was measured. Samples were taken at intervals and tested for RuBPCO activity and ATP concentration. RuBPCO activity and ATP decreased during photosynthesis in P_i deficient medium, but increased after addition of P_i. The increase was sharper with 5·0 mM P_i than with 0·3 mM P_i. O_2 evolution was low with no P_i and with 5·0 mM P_i, but it was high with 0·3 mM P_i. The results suggest that the decrease in RuBPCO activity could be related to the decrease in ATP concentration.

The ATP concentration and its relation to RuBP was investigated during photosynthesis by chloroplasts at various P_i concentrations and at 5 °C or 25 °C. ATP and RuBP concentrations were determined after the release of 2·5 μmol O_2 mg^{-1} chlorophyll (Table 1). When the P_i concentration in the medium was too low (no P_i added at 5 °C) or too high (1·8 mM P_i at 25 °C), the rate of photosynthesis was not high enough to release 2·5 μmol O_2 mg^{-1} chlorophyll (Mächler *et al.*, 1984). This was due to an imbalance between CO_2 assimilation and assimilate export. RuBP increased and ATP decreased when chloroplasts seemed to be deficient in P_i. This occurred at 25 °C in the absence of P_i and at 5 °C with 0·2 mM P_i. The increased RuBP under P_i deficient conditions seems to be the result of decreased RuBPCO activity, although it may be due partly to the influence of accumulated metabolites on the equilibrium of the exergonic RuBPCO reaction. The increase in RuBP when ATP decreased supports the hypothesis of a relationship between RuBPCO activity and ATP concentration.

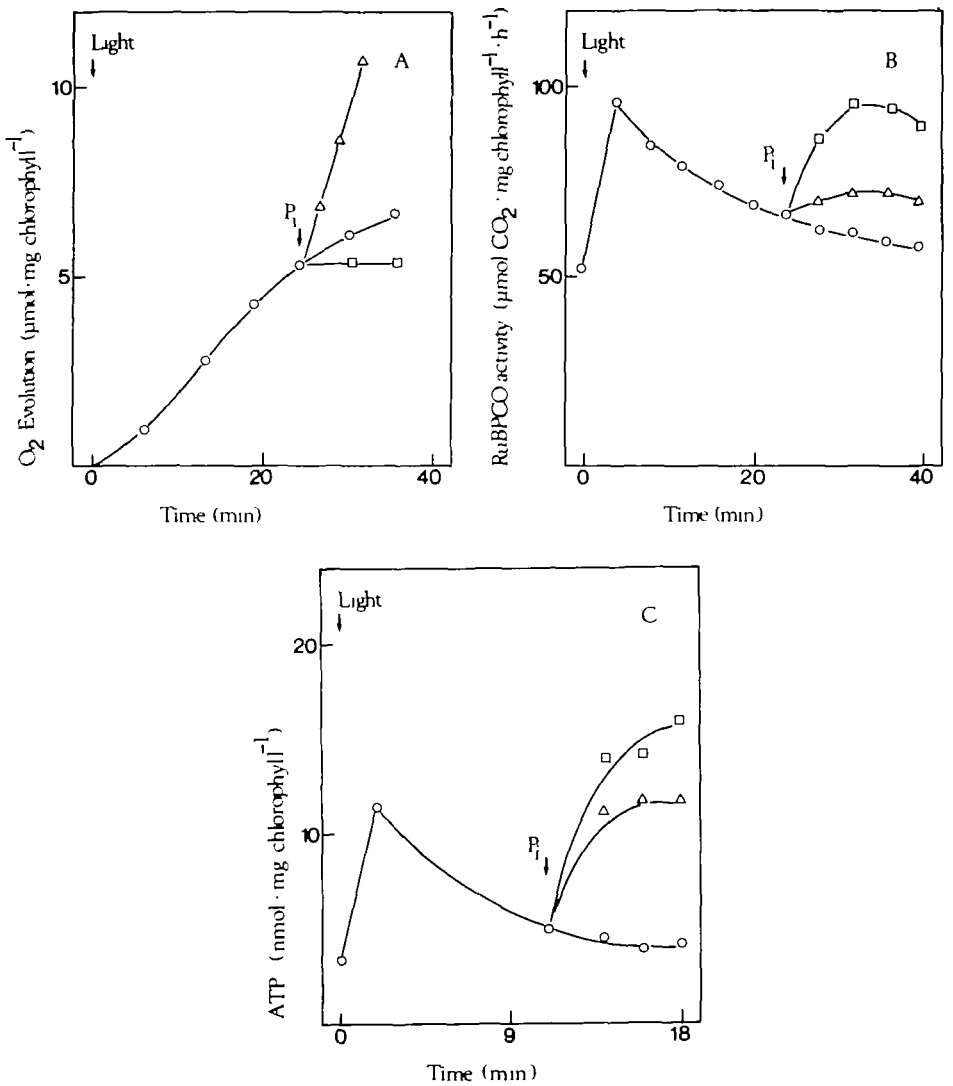

FIG. 2. Photosynthetic O_2 release (A), RuBPCO activity (B), and ATP content (C) of illuminated chloroplasts as influenced by P_i. Chloroplasts (20 μg chlorophyll cm^{-3} suspension) were pre-incubated and illuminated as described in Fig. 1 without P_i (○). After a period of photosynthesis 0·3 mM P_i (△), 5·0 mM P_i (□), or no P_i (○) was added. Samples were taken at intervals and tested for RuBPCO activity and ATP content. Oxygen evolution was monitored.

3. RuBPCO activity in chloroplasts during the photoreduction of various substrates differing in the consumption of ATP and P_i

Chloroplasts were pre-incubated in the dark for 20 min at 20 °C without P_i in 1·0 mM $NaHCO_3$ and with or without one of the following substrates: 1·0 mM oxaloacetate, 1·0 mM 3-phosphoglycerate, or 1·0 mM glycerate, and then illuminated (Fig. 3). The photoreduction of oxaloacetate, 3-phosphoglycerate, glycerate, and CO_2 was calculated to require 0, 2, 4, and 3 mol ATP and 0, 0, 2, and 1 mol P_i per mol O_2 released. RuBPCO was activated during the induction lag of photosynthesis when CO_2, oxaloacetate, or glycerate were the substrates. Thereafter, RuBPCO was inactivated during the photoreduction of CO_2 and glycerate but not

TABLE 1. *ATP and RuBP content of chloroplasts illuminated at 5 °C or 25 °C at various P_i concentrations*

Chloroplasts (60 μg chlorophyll cm^{-3} suspension) were pre-incubated in the dark for 20 min at 25 °C or for 40 min at 5 °C at various P_i concentrations and with 1·0 mM $NaHCO_3$ and then illuminated. 100 mm^3 samples of the chloroplast suspensions were taken when 2·5 μmol O_2 had been released, mixed immediately with 20 mm^3 of 3 N $HClO_4$ and centrifuged for 10 min at 3000 × *g*. 20 mm^3 of 1·0 M K_2HPO_4 was added to 100 mm^3 of supernatant and pH adjusted to 7·2 by adding 18 mm^3 of 3 N KOH. $KClO_4$ was sedimented and ATP and RuBP in the supernatant determined.

P_i in the medium	nmoles RuBP / mg chlorophyll		nmoles ATP / mg chlorophyll	
	5 °C	25 °C	5 °C	25 °C
No P_i in medium	—	23·9	—	2·4
0·2 mM P_i	19·4	14·4	3·6	6·6
0·6 mM P_i	14·9	14·6	6·2	7·4
1·8 mM P_i	13·4	—	8·6	—

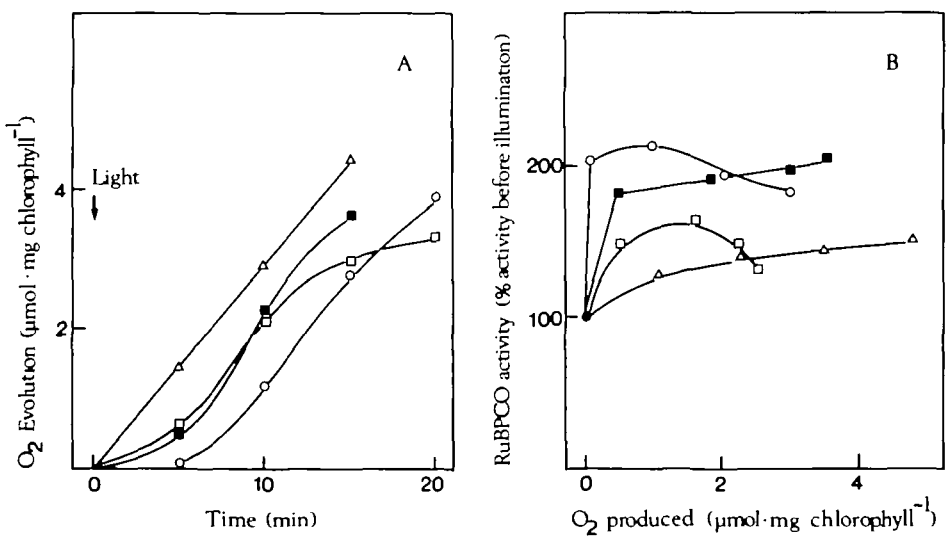

FIG. 3. Photosynthetic O_2 release (A) and RuBPCO activity (B) in chloroplasts during the photoreduction of various substrates differing in the consumption of ATP and P_i. Chloroplasts (90 μg chlorophyll cm^{-3} suspension) were pre-incubated in the dark for 20 min at 20 °C in 50 mM Hepes KOH (pH 7·6), 0·4 M sorbitol, 1·0 mM $NaHCO_3$, 1·0 mM EDTA and 200 units catalase cm^{-3} suspension with 1·0 mM oxaloacetate (■), 1·0 mM 3-phosphoglycerate (△), 1·0 mM glycerate (□) or no additional substrate (○). Chloroplasts were illuminated and samples taken for measurement of RuBPCO activity. RuBPCO activity before illumination was 26, 32, 23, and 31 μmol CO_2 mg^{-1} chlorophyll h^{-1} for oxaloacetate, 3-phosphoglycerate, glycerate, and no additional substrate, corresponding to 100%. RuBPCO activity was plotted against the sum of O_2 released.

during the photoreduction of oxaloacetate. The photoreduction of CO_2 and glycerate is associated with ATP and P_i consumption, whereas for the photoreduction of oxaloacetate neither ATP nor P_i is required. The results suggest a relationship between inactivation of RuBPCO and the consumption of ATP and P_i. Only decreased activation of RuBPCO occurred upon illumination in the presence of 3-phosphoglycerate, a substrate without

induction lag of photosynthesis. The photoreduction of 3-phosphoglycerate needs ATP, whereas no P_i is consumed. The decreased RuBPCO activity during photoreduction of 3-phosphoglycerate seems to be related to a decreased ATP concentration. RuBPCO activity was decreased, although no P_i was consumed suggesting that RuBPCO activity is related to ATP and not to P_i directly.

4. Purified RuBPCO as influenced by P_i or other metabolites when ATP is present

The inconsistency between the activation of RuBPCO by P_i *in vitro* and the lack of a direct relationship between P_i and RuBPCO activity *in vivo* was the incentive for the following experiments with purified RuBPCO.

Purified RuBPCO was pre-incubated in the presence of various compounds for 20 min at 20 °C and tested for RuBPCO activity (Table 2). RuBPCO was activated by P_i, 3-phosphoglycerate, and NADPH and inactivated by ribose-5-phosphate regardless of whether ADP was present or not. However, when ATP was present, RuBPCO was neither activated by P_i nor by 3-phosphoglycerate and only slightly by NADPH. Inactivation by ribose-5-phosphate was reinforced by ATP. ATP alone seemed to decrease RuBPCO activity to a lesser extent than when it was combined with a metabolite. The ATP effect seemed not to be due to sequestration of free Mg^{++} since it occurred not only with 2·0 mM Mg^{++} but also with 20 mM Mg^{++}, although to a lesser extent. The results suggest that the increased RuBPCO activity in chloroplasts in the presence of increased ATP and P_i concentrations is not due to direct effects of these compounds on the enzyme.

TABLE 2. *Influence of P_i and other metabolites on the activity of purified RuBPCO in the presence and absence of ATP and ADP*

Purified RuBPCO was incubated in 100 mM Tris HCl (pH 8·0), 1·0 mM $NaHCO_3$, 2·0 or 20·0 mM $MgCl_2$, 5·0 mM dithiothreitol, and effectors, as indicated in the table, for 20 min at 20 °C. RuBPCO activity was tested at 20 °C by adding 50 mm^3 of pretreated enzyme to 450 mm^3 of assay mixture. (n.d. = not determined, 3-PGA = 3-phosphoglycerate, R-5-P = ribose-5-phosphate).

Effectors added	RuBPCO activity (μmoles CO_2 min^{-1} mg^{-1} protein)				
	2·0 mM Mg^{++}			20·0 mM Mg^{++}	
	Control	2·0 mM ATP	2·0 mM ADP	2·0 mM ATP	2·0 mM ADP
Zero	0·162	0·130	n.d.	n.d.	n.d.
2·0 mM P_i	0·289	0·126	0·264	0·762	0·836
2·0 mM 3-PGA	0·275	0·145	0·238	0·696	0·780
2·0 mM R-5-P	0·096	0·043	0·088	0·197	0·364
2·0 mM NADP	n.d.	0·140	0·252	0·466	0·592
2·0 mM NADPH	n.d.	0·205	0·432	0·861	0·880

DISCUSSION

Inactivation of RuBPCO during photosynthesis seems to be related to a decreased P_i concentration in the stroma. RuBPCO activity seems to decrease when P_i in the stroma is consumed. RuBPCO is reactivated when P_i is added to the chloroplast suspension. However, P_i neither increases RuBPCO activity in the dark nor during the induction lag of photosynthesis upon illumination.

In illuminated chloroplasts ATP content, P_i concentration and RuBPCO activity seem to increase and decrease correspondingly. ATP seems to prevent P_i from activating RuBPCO

and thus a direct activation of RuBPCO by P_i is unlikely. The study suggests that RuBPCO activity is more closely related to ATP than to P_i.

It has been suggested earlier (Mächler *et al.*, 1984) that the ratios of ATP/ADP and dihydroxyacetone phosphate/3-phosphoglycerate decrease in illuminated chloroplasts when P_i is decreased. The decrease in RuBPCO activity, which occurs correspondingly, cannot be explained by a direct effect of these compounds on the enzyme. However, the pH in the stroma may decrease due to decreased ATP and accumulated 3-phosphoglycerate. This may be responsible for enzyme inactivation.

P_i deficiency in the chloroplast stroma occurs when CO_2 assimilation exceeds assimilate export. Photosynthetic products accumulate as organic phosphates in the stroma, resulting in decreased stromal P_i (Mächler *et al.*, 1984). The decrease in RuBPCO activity and photosynthesis, which occurs under these conditions, thus appears as a feedback inhibition.

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LITERATURE CITED

BHAGWAT, A. A., 1981. Activation of spinach ribulose 1,5-bisphosphate carboxylase by inorganic phosphate. *Plant Science Letters*, **23**, 197–206.

HELDT, H. W., CHON, C. J., and LORIMER, G. H., 1978. Phosphate requirement for the light activation of ribulose 1,5-bisphosphate carboxylase in intact spinach chloroplasts. *FEBS Letters*, **92**, 234–40.

LATZKO, E., and GIBBS, M., 1974. D-Ribulose-1,5-diphosphat und Pentosemonophosphat. In *Methoden der enzymatischen Analyse*. Ed. H. U. Bergmeyer. Verlag Chemie, Weinheim.

LORIMER, G. H., BADGER, M. R., and ANDREWS, T. J., 1976. The activation of ribulose 1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications. *Biochemistry*, **15**, 529–36.

—— —— —— 1977. D-Ribulose 1,5-bisphosphate carboxylase-oxygenase. *Analytical Biochemistry*, **78**, 66–75.

MÄCHLER, F., SCHNYDER, H., and NÖSBERGER, J., 1984. Influence of inorganic phosphate on photosynthesis of wheat chloroplasts. I. Photosynthesis and assimilate export. *Journal of Experimental Botany*, **35**, 481–7.

ROBINSON, S. P., MCNEIL, P. H., and WALKER, D. A., 1979. Ribulose bisphosphate carboxylase–lack of dark inactivation of the enzyme in experiments with protoplasts. *FEBS Letters*, **97**, 296–300.

SICHER, R. C., 1982. Reversible light-activation of ribulose bisphosphate carboxylase/oxygenase in isolated barley protoplasts and chloroplasts. *Plant Physiology*, **70**, 366–9.

STUMPF, D. K., and JENSEN, R. G., 1982. Photosynthetic CO_2 fixation at air levels of CO_2 by isolated spinach chloroplasts. Ibid. **69**, 1263–7.

WERDAN, K., HELDT, H. W., and MILOVANCEV, M., 1975. The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. *Biochimica et biophysica acta*, **396**, 276–92.