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EFFECTS OF INORGANIC PHOSPHATE ON THE PHOTOSYNTHETIC CARBON REDUCTION CYCLE IN EXTRACTS FROM THE STROMA OF PEA CHLOROPLASTS

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Summary

1. Turnover of the photosynthetic carbon reduction cycle has been demonstrated in chlorophyll-free reaction mixtures containing chloroplast stromal extract, as evidenced by the fixation of CO_2 following addition of small amounts of 3-phosphoglycerate.

2. The activity of the photosynthetic carbon reduction cycle in this system is inhibited by inorganic phosphate (P_i), with activity reduced to 50% by about 6.5 mM P_i. P_i also increased the lag period which elapsed before a steady rate of CO_2 fixation was obtained.

3. The effect of P_i on the rate of 3-phosphoglycerate reduction following the addition of substrate amounts of some cycle intermediates was investigated. Substantial inhibition was observed with fructose 1,6-bisphosphate, sedoheptulose 1,7-bisphosphate and erythrose 4-phosphate as substrates. P_i also affected the activity of ribulose-bisphosphate carboxylase, with stimulation at P_i concentrations below 2.5 mM and inhibition at higher concentrations.

4. The results showed that the sedoheptulose bisphosphatase reaction is inhibited more strongly by P_i than the fructose bisphosphatase reaction.

5. It is concluded that the previously established inhibitory effects of P_i on photosynthesis by intact isolated chloroplasts may be partly due to these inhibitory effects of P_i on the reactions of the photosynthetic carbon reduction cycle.

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Introduction

Inorganic phosphate is a substrate for photosynthetic carbon fixation by isolated chloroplasts, which require an external concentration in the range of 0.1—0.5 mM P_i for maximum activity [1]. At higher concentrations (1—10 mM), however, P_i is a well-established inhibitor of CO_2 fixation by isolated intact chloroplasts [1,2]. In addition to influencing the rate of photosynthesis, the concentration of P_i external to the chloroplast determines the fate of fixed carbon by controlling the alternative pathways of internal starch synthesis or export of triose phosphate and 3-phosphoglycerate to the external medium [2]. Starch synthesis is favoured by low P_i concentrations in isolated chloroplasts and in leaf tissue, while high concentrations favour the mobilisation of starch reserves [3,4].

The mechanism by which external P_i influences photosynthesis has been attributed to the operation of the phosphate translocator, an antiport located in the inner membrane of the chloroplast envelope which facilitates a rapid counter-exchange of P_i, 3-phosphoglycerate and triose phosphate [5]. Sub-optimal P_i concentrations result in a build-up of triose phosphates and 3-phosphoglycerate within the chloroplast and consequent starch synthesis whereas high P_i concentrations result in depletion of these intermediates with consequent inhibition of photosynthetic carbon reduction cycle (Benson-Calvin cycle) turnover and of photosynthesis [2].

It has previously been recognised, however, that the inhibitory action of high P_i concentrations may be partly due to effects unconnected with the P_i translocator [2,3]. While some of these effects may be associated with the reported interference by P_i in the light activation of some photosynthetic carbon reduction cycle intermediates [6], direct effects of P_i on the activity of enzymes of the cycle remain a possibility. It seemed, therefore, that if a membrane-free reaction mixture containing chloroplast extract could be devised which would exhibit CO_2 fixation and Benson-Calvin cycle turnover, then such a system might prove to be useful for measuring the effects of P_i on the activity of the cycle as a whole and on its component reactions.

Methods

Peas (*Pisum sativum*, Massey Gem, Arthur Yates & Co., Sydney, Australia) were grown in vermiculite for 9 to 11 days in a glasshouse, under natural lighting conditions.

Pea leaves were harvested, washed in distilled water and disrupted by a Polytron blender in 200 ml medium 1 (0.33 M glucose, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 11 mM NaCl, 7.5 mM sodium isoascorbate, adjusted to pH 6.5 with KOH). Chloroplasts were isolated by centrifuging for 30 s at 2500 × *g*. The chloroplasts were washed by resuspension in 100 ml medium 2 (0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 0.2% bovine serum albumin, 0.5 mM dithiothreitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), adjusted to pH 8.0 with KOH) and centrifuged for 30 s at 2500 × *g*. The resulting chloroplasts were usually 90—95% intact, measured by ferricyanide reduction [7].

For the preparation of chloroplast extract, chloroplasts were osmotically shocked by resuspension in a 1/13 dilution of resuspension medium and centrifuged for 15 min at 9000 × *g*. The resulting pellet of envelope-free chloroplasts was retained for chlorophyll estimation by the method of Wintermans and De Mots [8]. The supernatant, (chloroplast stromal extract), was subjected to concentrating dialysis (MicroProDiCon, Biomolecular Dynamics, Oregon, U.S.A.). A 10 000 molecular weight cut-off membrane was used and the chloroplast extract dialysed against 1 l 10 mM Hepes, 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$ and 0.5 mM dithiothreitol, adjusted to pH 8.0 with KOH. The concentrating dialysis was maintained for 4 h at 2° C and the volume of the chloroplast extract was reduced by a factor of 4. The concentrated chloroplast extract was immediately subdivided into 250-μl aliquots in small plastic tubes and placed in liquid N_2. Assays on a rethawed sample of chloroplast extract were completed within 2 h.

Protein was estimated by the Biuret method [9], and inorganic phosphate was determined by the method of Lowry and Lopez [10].

Pyridine nucleotide oxidation was measured in reaction mixtures at 25° C by following the rate of decrease in absorbance at 340 nm, using a spectrophotometer (Varian Model 635). Each reaction mixture contained 40 mM Hepes, 8 mM KCl, 0.8 mM EDTA, 20 mM $MgCl_2$, 1 mM NADPH, 10 mM dithiothreitol, 4 mM ATP, 10 mM phosphocreatine, 1.1 units creatine kinase (EC 2.7.3.2), 10 mM $NaHCO_3$ and chloroplast extract containing 1.3—2.7 mg protein in a total volume of 500 μl at pH 8.0. Where NADH oxidation was measured, NADPH was replaced with 1 mM NADH plus 1.25 units of yeast NAD^+-glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12) and of phosphoglycerate kinase (EC 2.7.2.3). In the case of all assays for ribose 5-phosphate- or ribulose 1,5-bisphosphate-dependent NADH oxidation, the chloroplast extract was incubated before assay in 20 mM $MgCl_2$, 10 mM $NaHCO_3$ and 10 mM dithiothreitol for 5 min at 25° C.

In experiments where CO_2 fixation was determined, the same concentrations of reactants were present, but sodium bi[^{14}C]carbonate (0.1 Ci · mol^{-1}) was used and the total reaction volume was increased to 750 μl. 20-μl samples were withdrawn at intervals and injected into 100 μl CH_3OH in a scintillation vial. 10 μl formic acid was then added and the contents of the vial evaporated to dryness before before the addition of scintillation cocktail. Radioactivity was determined in a Packard C-2425 scintillation counter. Control samples, for measuring total radioactivity, were taken at the commencement and conclusion of each experiment by injecting 20-μl samples into scintillation vials containing 10 μl of Carbosorb 2 (Packard) in addition to scintillation cocktail. Quench compensation was performed by the internal standard method.

Biochemicals were purchased from the Sigma Chemical Company and were all of high purity, except for erythrose 4-phosphate (80%) and sedoheptulose 7-phosphate (85%) which was supplied as the barium salt, and was converted to the sodium salt by passage through a small column of ion-exchange resin (Na^+ Amberlite IR-20) before use.

Results

The addition of 140 nmol 3-phosphoglycerate to reaction mixtures resulted in the rapid oxidation of an equal amount of NADPH which was complete within about 1 min (Fig. 1). No carbon fixation occurred during this period. After a brief lag, however, the rate of NADPH oxidation increased again until a linear rate was reached, which was maintained for at least 15 min (upper trace). This additional NADPH oxidation was accompanied by the onset of carbon fixation, with one atom of carbon fixed per two molecules NADPH oxidised.

If the reaction mixture contained, in addition, 10 mM P_i (Fig. 1, lower trace), the initial oxidation of 3-phosphoglycerate occurred at much the same rate, but the lag time before additional oxidation commenced was greatly extended. The final rates of NADPH oxidation and carbon fixation achieved were much less than in the absence of added P_i. There was not exact correspondence between the amount of carbon fixed and half the additional amount of NADPH oxidised under these conditions, although the displacement between the two time-courses was fairly constant and the final rates achieved similar. When these experiments were repeated, but substituting NADH plus the yeast enzymes phosphoglycerate kinase and NAD^+-glyceraldehyde-phosphate dehydrogenase for NADPH, essentially identical results were obtained.

The effect of a range of different P_i concentrations on the final rate of NADH oxidation is shown in Fig. 2. From that graph, a 50% inhibition of NADH oxidation would be caused by 6.5 mM P_i. Although the degree of inhibition varied somewhat with different chloroplast extract preparations, substantial inhibition was always observed by P_i concentrations of 1 mM or higher.

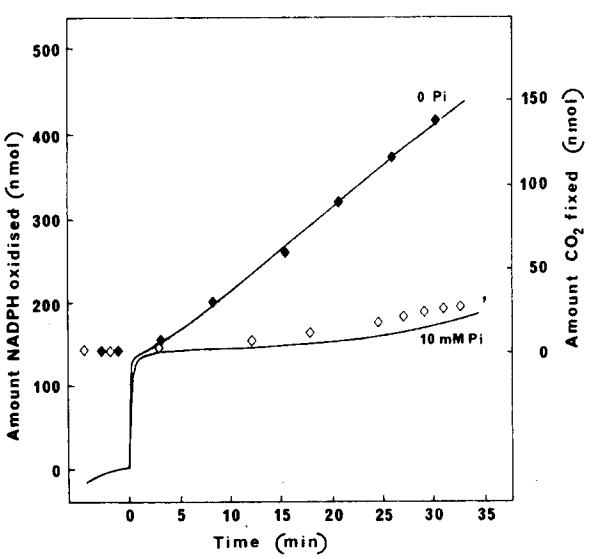


Fig. 1. Simultaneous measurements of NADPH oxidation (recorder trace) and carbon fixation (points) in reaction mixtures following the addition of 140 nmol 3-phosphoglycerate in the absence of P_i (upper trace, ♦) and with 10 mM P_i (lower trace, ◇). The rates of NADPH oxidation over the final linear portions of the recorder traces were 64 and 18 nmol NADPH oxidised per mg protein per h, respectively.

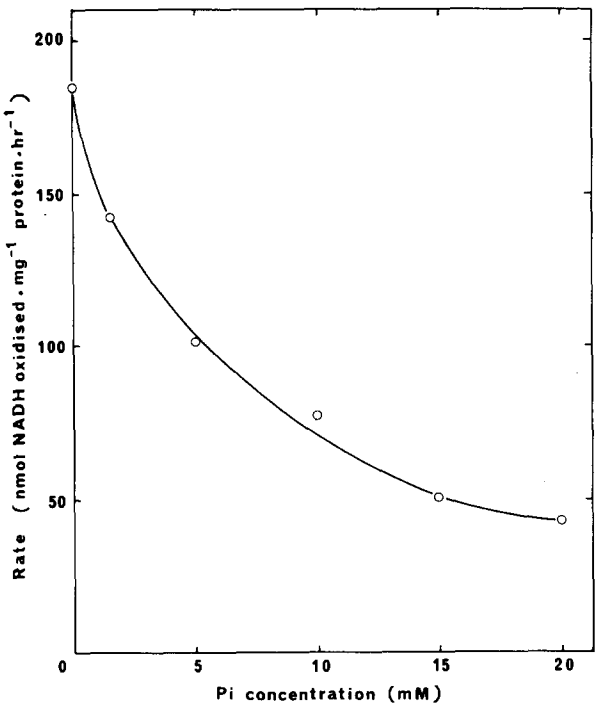


Fig. 2. Effect of P_i on NADH oxidation by reaction mixtures following the reduction of 100 nmol 3-phosphoglycerate. Rates determined from the final linear portion of the time-course (see Fig. 1).

The effect of P_i on the oxidation of NADH by a substrate amount of ribose 5-phosphate is shown in Fig. 3a. The chloroplast extract in this case was pre-incubated to activate fully the ribulose-bisphosphate carboxylase (EC 4.1.1.39) (see Methods). Under the conditions used, the activity of the carboxylase is rate-limiting for NADH oxidation and the system becomes an assay for this enzyme [11,12]. Low concentrations of P_i increased the activity of ribulose-bisphosphate carboxylase with peak activity at 1 mM P_i. At P_i concentrations above 1 mM, however, the activity was inhibited with maximum inhibition (about 50%) at 10 mM P_i. Almost identical results were obtained when the experiment was repeated substituting ribulose 1,5-bisphosphate as substrate instead of ribose 5-phosphate (data not shown). With fructose 1,6-bisphosphate as substrate, however, the rate of NADH oxidation was inhibited over the entire range of P_i concentrations tested, with greatest inhibition by 20 mM P_i. Although 3-phosphoglycerate-dependent NADH oxidation was slightly inhibited by P_i, the rates obtained were much higher than with the other substrates, and it is clear that the effects of P_i with ribose 5-phosphate or fructose 1,6-bisphosphate as substrates were not due to any effect on the reaction sequence converting 3-phosphoglycerate to glyceraldehyde 3-phosphate. The effects of P_i on NADPH oxidation by substrate amounts of some Benson-Calvin cycle intermediates are shown in Fig. 3b. As before, 3-phosphoglycerate-dependent oxidation was the least sensitive to P_i and the rates obtained much higher than with the other substrates. The activities with fructose 1,6-bisphosphate, sedoheptulose 1,7-bisphosphate and erythrose 4-phosphate were all

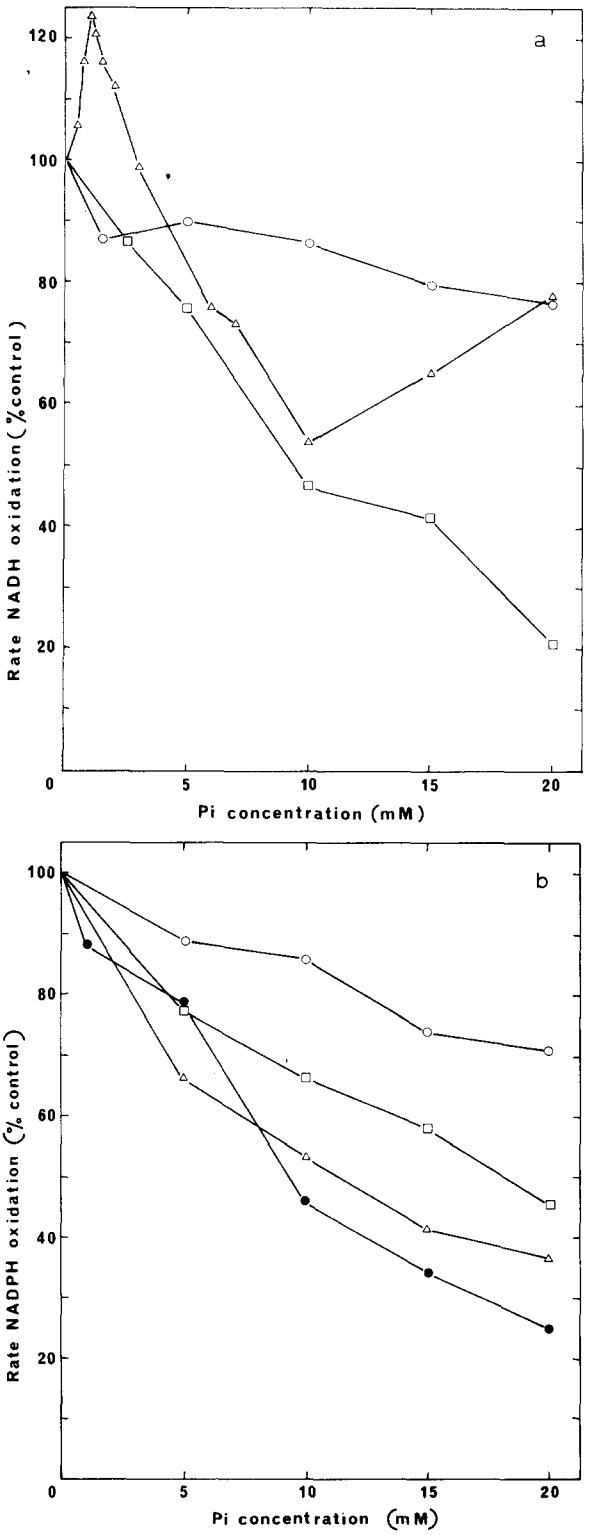


Fig. 3. Effect of P_i on relative rates of NAD(P)H oxidation by reaction mixtures following the addition of 1 μmol 3-phosphoglycerate. Rates determined from the linear portion of the time-course (see Fig. 4). Control rates (in absence of added P_i) are given as μmol NAD(P)H oxidised per mg protein per h with the following substrates. (a) NADH oxidation: ○———○, 3-phosphoglycerate (69.0); △———△, ribose 5-phosphate (22.8); □———□, fructose 1,6-bisphosphate (2.77). (b) NADPH oxidation: ○———○, 3-phosphoglycerate (8.48); □———□, fructose 1,6-bisphosphate (1.37); △———△, sedopheptulose 1,7-bisphosphate (2.31); ●———●, erythrose 4-phosphate (0.57).

inhibited by P_i (in order of increasing severity). Erythrose 4-phosphate (1 μmol) was added together with 125 nmol 3-phosphoglycerate and the rate of NADPH oxidation was measured after the initial rapid oxidation of 125 nmol NADPH, since the activity with erythrose 4-phosphate alone was low.

In addition to inhibiting the rate of NAD(P)H oxidation, P_i increased the lag period before a constant rate of oxidation was achieved. This is seen in Fig. 1 where 10 mM P_i greatly increased the lag period before CO_2 fixation commenced and NADPH oxidation recommenced, following the initial reduction of a small amount of 3-phosphoglycerate. Recorder traces of NADPH oxidation with substrate amounts of sedoheptulose 1,7-bisphosphate are shown in Fig. 4. The time which elapsed before a constant rate of NADPH oxidation was achieved, increased progressively from about 4 min in the absence of added P_i to about 12 min in the presence of 20 mM P_i.

The effects of 10 mM P_i on the rates of NADH oxidation with different substrates are compared in Table I. While the rate with sedoheptulose 1,7-bisphosphate is inhibited more by P_i than with fructose 1,6-bisphosphate, that with sedoheptulose 7-phosphate is much less sensitive to P_i than with fructose 6-phosphate. This shows that the reaction catalysed by sedoheptulose bisphosphatase (EC 3.1.3.37) is more sensitive to P_i than the fructose bisphosphatase reaction (EC 3.1.3.11). When the sedoheptulose bisphosphatase is bypassed by supplying its product, sedoheptulose 7-phosphate, the rate of NADH oxidation is then least inhibited by 10 mM P_i.

It is interesting that the activities with sedoheptulose 1,7-bisphosphate and sedoheptulose 7-phosphate are substantially higher than those with fructose

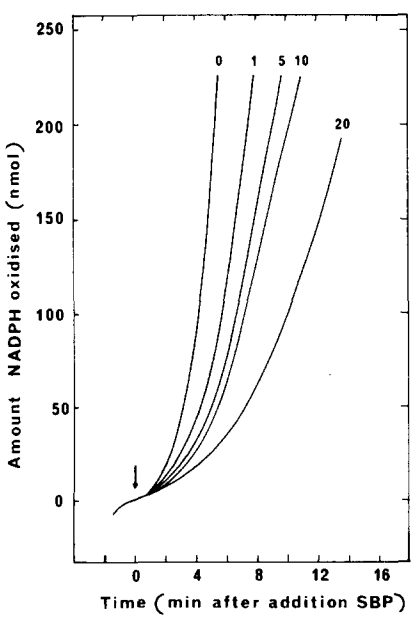


Fig. 4. Recorder traces of NADPH oxidation following the addition (arrow) of 0.5 μmol sedoheptulose 1,7-bisphosphate (SBP) to reaction mixtures containing various concentrations of P_i. The number adjacent to each trace is the P_i concentration (mM).

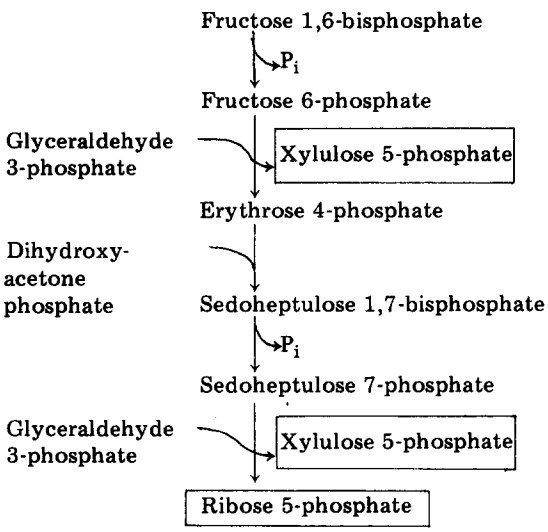
TABLE I

THE EFFECT OF P_i ON RATES OF NADH OXIDATION WITH DIFFERENT SUBSTRATES

NADH oxidation was measured in the reaction mixture described in Methods. The concentration of each substrate was 1 mM.

Substrate	0 mM P_i mean control rate (μmol NADH oxidised per mg protein per h)	10 mM P_i % control (mean ± S.D., 4 determinations)
Sedoheptulose 1,7-bisphosphate	3.07	38 ± 4
Sedoheptulose 7-phosphate	4.71	70 ± 4
Fructose 1,6-bisphosphate	1.95	61 ± 10
Fructose 6-phosphate	2.01	40 ± 4

1,6-bisphosphate and fructose 6-phosphate respectively under the same conditions (Table I). This can be attributed to the formation, from sedoheptulose 7-phosphate, of two out of the three pentose phosphate molecules synthesised during each turn of the Benson-Calvin cycle (Scheme I), while fructose 6-phos-



Scheme I.

phate is the immediate precursor of one pentose phosphate molecule. Since the utilisation of ribose 5-phosphate is relatively rapid (Fig. 3a), the rate of NADH oxidation can be expected to be proportional to the rate of pentose phosphate generation.

Specific and general phosphatase action on the many phosphate esters present in these reaction mixtures might be expected to result in significant net release of P_i during the assays. To establish whether large net increases in the concentration of P_i were occurring, the concentration of P_i was measured at intervals during substrate-dependent NADPH oxidation (Table II). The amount of P_i released during NADPH oxidation with sedoheptulose 1,7-bisphosphate was lower than the expected P_i/NADPH ratio of 1.25 (assuming that one sedoheptulose 1,7-bisphosphate molecule results, via the formation of two pentose

TABLE II

RELEASE OF INORGANIC PHOSPHATE DURING SUBSTRATE-DEPENDENT OXIDATION OF NADPH

NADPH oxidation and P_i concentration were measured in reaction mixtures following the addition of 1.0 μmol substrate. The concentration of P_i in the reaction mixture was then determined.

Substrate	Time (min)	NADPH oxidised (nmol)	P_i concentration (mM)	P_i released (nmol)	Ratio P_i released/ NADPH oxidised
Sedoheptulose 1,7-bisphosphate	0	0	0.12	0	—
	5	20	0.15	15	0.75
	10	95	0.31	95	1.00
Fructose 1,6-bisphosphate	0	0	0.12	0	—
	5	35	0.22	50	1.43
	10	75	0.33	105	1.40

phosphate molecules in the formation of four glyceraldehyde 3-phosphate molecules; thus four NADPH are oxidised and five P_i released). P_i release with fructose 1,6-bisphosphate was close to the expected ratio of 1.5 (assuming that one fructose 1,6-bisphosphate molecule results, via the formation of one pentose phosphate in the formation of two glyceraldehyde 3-phosphate molecules; thus two NADPH are oxidised and three P_i released). The initial P_i concentration (0.12 mM) in the reaction mixtures was largely due to residual P_i in the chloroplast extract, but the final concentrations of about 0.3 mM were low compared with the range of added P_i concentrations (0—20 mM).

The average protein to chlorophyll ratio of the isolated chloroplasts used to prepare chloroplast extract was 24.0 ± 4.1 (10). The best rate of NADH oxidation observed during Benson-Calvin cycle turnover following the addition of catalytic amounts of 3-phosphoglycerate was 184 nmol · mg^{-1} protein · h^{-1} (Fig. 2), equivalent to 92 nmol CO_2 fixed per mg protein per h, which therefore corresponded to 2.2 μmol CO_2 fixed per mg chlorophyll per h, or about 2% of the rate of photosynthesis typically observed in plant leaves [13]. Much higher activities were observed with substrate amounts of cycle intermediates (e.g. Table I). The activities measured with freshly prepared chloroplast extract were not significantly affected by freezing the extract in liquid N_2 and then rethawing. The loss of activity during storage in liquid N_2 was less than 1% per day for all measured activities.

Discussion

Reconstituted chloroplast systems [14], containing chloroplast stromal extract and thylakoids, are capable of photosynthetic carbon reduction cycle turnover [15] and autocatalysis [16], and have yielded much information on the regulation of photosynthesis. In the experiments reported here, the thylakoids were replaced by a substrate amount of NADPH (or by NADH plus the appropriate enzymes) and an artificial ATP-regenerating system (phospho-

creatine and creatine kinase). Similar reaction mixtures have been shown to reduce quantitatively 3-phosphoglycerate [17] and to drive ribulose 1,5-bisphosphate- and ribose 5-phosphate-dependent NADH oxidation with a stoichiometry of two NADH oxidised per CO_2 fixed [11]. Here, the demonstration of CO_2 fixation following the addition of small amounts of 3-phosphoglycerate to reaction mixtures shows that turnover of the photosynthetic carbon reduction cycle was occurring, since 3-phosphoglycerate is the product of the carboxylation reaction [15]. It is therefore possible to study the activity of all or parts of the Benson-Calvin cycle in this chlorophyll-free system and to monitor the progress of these reactions by NADPH or NADH oxidation as well as by CO_2 fixation.

Both NADPH oxidation, dependent entirely on the endogenous chloroplast stroma enzymes, and NADH oxidation coupled to added NAD^+-glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase from yeast were utilised in these experiments. While similar results were obtained in both cases at low rates of NADPH or NADH oxidation, at high rates of photosynthetic carbon reduction cycle activity (e.g. with ribose 5-phosphate as substrate), the rate of 3-phosphoglycerate production may exceed the capacity of the NADP-linked enzymes to reduce it. The replacement of NADPH by NADH plus high activities of the yeast enzymes then increases greatly the rate of 3-phosphoglycerate utilisation, ensuring that other reactions further back in the cycle remain rate-limiting for NADH oxidation.

The concentration of P_i in the stroma of isolated spinach chloroplasts decreases from 13 mM to 6 mM during the first 10 min of illumination, while the amount of organically bound P_i increases by a similar amount [18]. An earlier study of P_i in chloroplasts in the leaves of a range of plants found concentrations ranging from 4 to 25 mM [19]. The 0—20 mM P_i range examined in the present work seems therefore to be representative of the in vivo conditions.

The results show that, while a number of reactions of the Benson-Calvin cycle are inhibited by P_i, the most sensitive is sedoheptulose bisphosphatase. The fructose bisphosphatase reaction is not as strongly inhibited by P_i (Fig. 3b and Table I), but results in the immediate formation of one pentose phosphate molecule, permitting some cycle turnover independently of the sedopheptulose bisphosphatase reaction (Scheme I). Conversely, the utilisation of erythrose 4-phosphate, which has a slightly higher sensitivity to P_i, is dependent on the sedoheptulose bisphosphatase reaction for the formation of pentose phosphates. Both fructose and sedoheptulose bisphosphatases have been implicated in the regulation of the photosynthetic carbon reduction cycle by light-dependent changes in Mg^{2+} concentration [20,21] and pH in the stroma [22]. Steup et al. [3], however, have also presented evidence that sedoheptulose bisphosphatase in photosynthesising intact spinach chloroplasts is inhibited more by externally supplied P_i than fructose bisphosphatase.

Although the light activation of ribulose-bisphosphate carboxylase in isolated spinach chloroplasts has been shown to require the presence of 1 mM P_i in the medium [23], the complex effects of P_i on the carboxylase reported here are likely to be the result of direct modulation of the activity of the enzyme rather than changes in the proportion of active and inactive forms of

the enzyme. This conclusion is based on the fact that the assays were conducted with chloroplast extract pretreated under conditions that fully activate the carboxylase and that the initial rates of activity remained constant for several minutes [24]. Paulsen and Lane [25] have reported an inhibition of this enzyme by P_i, although their procedures did not include precautions to activate the carboxylase fully before assay. These effects of P_i on the carboxylase may be significant in vivo, since its activity is then limited by the low CO_2 concentrations within the leaf and this reaction may be a rate-limiting factor in photosynthesis [13].

The well-known induction effects in plant photosynthesis during illumination after a long dark period have previously been interpreted in terms of the autocatalytic nature of the Benson-Calvin cycle [2]. The present results show that P_i both inhibits the cycle and lengthens the lag time before the rate of carbon fixation reaches a steady level, while previous work shows that the amount of P_i in the chloroplast stroma decreases during initial illumination [17,26]. It is already established that P_i has a central role in the regulation of starch synthesis [3,4] and the export of fixed carbon from the chloroplast via the phosphate translocator in the inner membrane [5]. If our present conclusions are correct, the regulatory role of the stromal P_i concentration is extended to the direct control of photosynthetic carbon reduction cycle activity and the mediation of induction effects.

Acknowledgements

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