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THE REDUCTION OF 3-PHOSPHOGLYCERATE BY RECONSTITUTED CHLOROPLASTS AND BY CHLOROPLAST EXTRACTS

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SUMMARY

1 The rate of 3-phosphoglycerate reduction in extracts from spinach chloroplasts, assayed by spectrophotometric measurement of 3-phosphoglycerate-dependent NADPH oxidation, was strongly inhibited by ADP AMP was much less inhibitory

2 Oxygen evolution by reconstituted chloroplasts with 3-phosphoglycerate as substrate was also inhibited by the addition of ADP or following uncoupling by added NH_4Cl

3 In all cases the inhibitory effects of ADP were reversed by addition of phosphocreatine and creatine phosphokinase activity

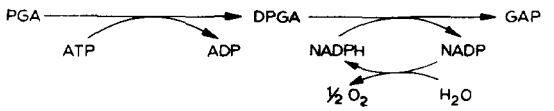
4 The stoichiometry of 3-phosphoglycerate reduction to NADPH oxidation in chloroplast extracts was 1 1 and there was negligible turnover of the Benson–Calvin cycle in either chloroplast extracts or in reconstituted chloroplasts under the particular conditions employed

5 The maximum rate of 3-phosphoglycerate-dependent O_2 evolution by reconstituted chloroplasts was ultimately limited by NADP reduction and photophosphorylation, and was similar to the maximum rate of oxygen evolution under optimal conditions by intact chloroplasts In the presence of sufficient ADP phosphorylating activity, the rate of enzymic 3-phosphoglycerate reduction was relatively high The inhibition of this reaction by ADP may represent a control mechanism in photosynthesis

INTRODUCTION

The reduction of 3-phosphoglycerate to triose phosphate is a singularly important reaction in photosynthesis which links the photochemical events in the thylakoid membranes to the so-called "dark biochemistry" of the Benson–Calvin cycle in the chloroplast stroma compartment Isolated intact chloroplasts (type A) are capable of high rates of O_2 evolution with 3-phosphoglycerate as substrate [1] Preparations of washed envelope-free chloroplasts (type D [1]) however, will evolve O_2 with 3-phosphoglycerate only when supplied with a chloroplast extract (stromal

protein) and sufficient ferredoxin, NADP and ATP to give a "reconstituted" chloroplast system [2] Because the chloroplast envelope is impermeable or relatively impermeable to most intermediates and cofactors of the carbon fixation cycle [3, 4], the reconstituted system is particularly useful for studies involving the experimental manipulation of the concentrations of these compounds Oxygen evolution by the reconstituted system is essentially a Hill reaction in which the natural oxidant, NADP, is reduced by electrons from water and reoxidised by 1,3-diphosphoglycerate (DPGA)



where PGA means 3-phosphoglycerate and GAP, glyceraldehyde-3-*P*

This reaction proceeds in the light utilising either exogenous 3-phosphoglycerate or that produced by carboxylation of ribulose 1,5-diphosphate Under some conditions, the rate of CO_2 fixation can exceed the rate of O_2 evolution in the reconstituted chloroplast system [5], implying rate limitation by one or more reaction steps in the above sequence

Parallel measurements of 3-phosphoglycerate reduction in stromal extracts obtained by osmotic shock of intact chloroplasts, and of 3-phosphoglycerate-dependent O_2 evolution by reconstituted chloroplasts are reported here The apparent activity of NADP-specific glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate NADP oxidoreductase (phosphorylating), EC 1 2 1 13), was studied in freshly prepared and unfractionated chloroplast extract in order to identify those factors determining the apparent activity of this enzyme which are likely to operate in vivo

MATERIALS AND METHODS

Preparation of chloroplasts

Spinach (*Spinacia oleracea* var True Hybrid 102, Arthur Yates and Co , N S W , Australia) was grown in aerated nutrient solution in a glasshouse under supplementary artificial lighting The conditions and composition of nutrient solution were similar to those used by Randall and Bouma [6] for growth in their control medium Intact chloroplasts were prepared as described previously [7], except that the isolation medium was 0 33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$ and 2 mM D-*iso*-ascorbate, adjusted to pH 6 5 with HCl The composition of the chloroplast resuspending medium was 0 33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, adjusted to pH 7 6 with KOH For the preparation of chloroplast extract [5], the pellet of intact chloroplasts (containing 2 5–3 0 mg chlorophyll, measured by the method of Arnon [8]) was instead resuspended in 5 5 ml of a solution containing 1/25 dilution of resuspending medium and 5 mM dithiothreitol After stirring for 2 min (to allow rupture of the chloroplast envelope membranes by osmotic shock) the suspension was centrifuged for 10 min at 13 000 × *g* The supernatant (chloroplast extract) was free of chlorophyll and contained 3 9–4 7 mg protein per ml, measured by the biuret

method [9] after precipitation with trichloroacetic acid The precipitate (envelope-free chloroplasts) was resuspended in full-strength medium as before The volume of chloroplast extract per unit chlorophyll was calculated from the total volume of chloroplast extract and the total chlorophyll in the envelope-free chloroplasts

Spectrophotometric assay of NADPH oxidation by chloroplast extracts

The basic reaction mixture contained 0 33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, 10 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 1 mM NADPH, ATP and $MgCl_2$ as specified, plus 50 μl chloroplast extract in a final volume of 0 5 ml at pH 7 6 in a 2-mm optical path cuvette Changes in optical density at 340 nm were measured at 20 °C using a split-beam spectrophotometer and a recorder calibrated to give direct readings in μmoles NADPH Primary standards were solutions freshly prepared from preweighed vials of desiccated NADH (Sigma Chemical Co) Reaction mixtures were preincubated for 5 min before the addition of 3-phosphoglycerate

Measurement of 3-phosphoglycerate-dependent oxygen evolution by reconstituted chloroplasts

Oxygen evolution by twin reaction mixtures containing reconstituted chloroplasts illuminated with red light was measured as before [10] Normally each reaction mixture contained envelope-free chloroplasts (200 μg chlorophyll), chloroplast extract (equivalent to 200 μg chlorophyll), 0 33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, 10 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM $NaHCO_3$, 2 mM ATP, 0 125 mM NADP and 44 μg spinach ferredoxin in a final volume of 2 ml at pH 7 6 and 20 °C

Reagents

All inorganic compounds used were of analytical reagent grade Biochemicals were of the highest purity obtainable from the Sigma Chemical Co Fresh solutions of NADPH and creatine phosphokinase were prepared each day from desiccated solid Phosphoglycerate kinase was dialysed before use to remove $(NH_4)_2SO_4$ Spinach ferredoxin was prepared by the method of Rao et al [11]

RESULTS AND DISCUSSION

3-Phosphoglycerate-dependent NADPH oxidation by chloroplast extracts

Recorder traces of NADPH oxidation in reaction mixtures containing chloroplast extract are shown in Fig 1 In the presence of 10 mM $MgCl_2$ (Fig 1a, trace A), there was an initial rapid phase of NADPH oxidation during the first 15 s, partially obscured by the excursion of the trace during mixing The rate of NADPH oxidation then declined progressively The extent of the decline in activity was somewhat variable between different chloroplast extract preparations, and was less marked in the presence of lower Mg^{2+} or higher 3-phosphoglycerate concentrations

In the presence of an ATP-regenerating system (Fig 1a, trace B), the rate of NADPH oxidation was increased and became linear after 2 min The phosphocreatine/creatine phosphokinase system used here may be regarded as an analogue of photophosphorylation If the concentration of $MgCl_2$ was increased to 15 mM and

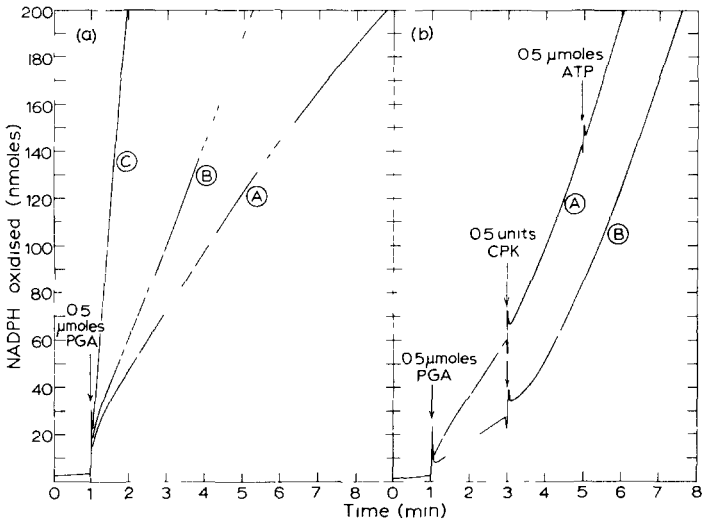
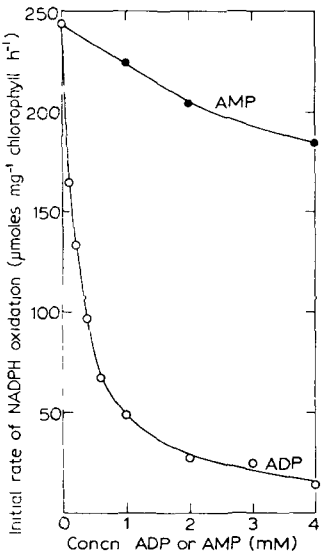


Fig 1 Recorder traces of 3-phosphoglycerate-dependent NADPH oxidation by chloroplast extract (a) Basic reaction mixture (see Materials and Methods) plus 2 mM ATP In addition Expt A contained 10 mM $MgCl_2$, Expt B contained 10 mM $MgCl_2$, 5 mM phosphocreatine and 0 5 unit creatine phosphokinase activity, Expt C contained 15 mM $MgCl_2$, 5 mM phosphocreatine and 2 units creatine phosphokinase activity (b) Basic reaction mixture plus 2 mM ATP, 5 mM phosphocreatine and 5 mM $MgCl_2$ In addition Expt B contained 1 mM ADP Additions of 3-phosphoglycerate (PGA) creatine phosphokinase (CPK) and ATP where indicated

the amount of creatine phosphokinase increased from 0 5 to 2 units (Fig 1a, trace C) the rate was much more rapid and was linear for the first 30 s after 3-phosphoglycerate addition The slight decline in rate towards the top of trace C is due to depletion of the 3-phosphoglycerate substrate The results of Fig 1a suggested that the reduction of 3-phosphoglycerate to glyceraldehyde 3-phosphate was inhibited by ADP formed in the 3-phosphoglycerate kinase reaction In the absence of the ATP-regenerating system, the addition of excess phosphoglycerate kinase (approx 100 activity units) either had no effect or was slightly inhibitory, confirming an earlier observation [12] In its presence there was a slight stimulation (up to 4 %) It was, therefore, concluded that the enzymic phosphoglycerate kinase activity in the chloroplast extract was not rate-limiting However, it is not possible to distinguish from these experiments whether the inhibition by ADP is a mass action effect on the endergonic phosphoglycerate kinase reaction or inhibition of enzymic NADP-specific 3-phosphoglycerate dehydrogenase activity

The inhibitory effect of ADP could be fully reversed by the ATP-regenerating system (Fig 1b) The rate of NADPH oxidation was inhibited by more than 50 % in a reaction mixture containing 1 mM ADP (trace B), but, after the addition of creatine phosphokinase to both reaction mixtures, accelerated until it equalled that of the reaction mixture containing no added ADP (trace A)

The effect of increasing ADP concentration on the rate of 3-phosphoglycerate-dependent NADPH oxidation in reaction mixtures without the ATP-regenerating system is shown in Fig 2 Low concentrations of ADP (less than 1 mM) caused



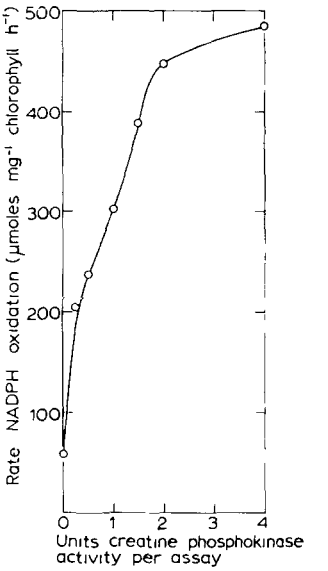


Fig 2 Initial rate of 3-phosphoglycerate-dependent NADPH oxidation by chloroplast extract Basic reaction medium (see Materials and Methods) plus 4 mM ATP 1 mM $MgCl_2$ and ADP or AMP as specified Reactions started by addition of 0 5 μmole 3-phosphoglycerate

Fig 3 Initial rate of 3-phosphoglycerate-dependent NADPH oxidation by chloroplast extract Basic reaction mixture (see Materials and Methods) plus 2 mM ATP, 5 mM phosphocreatine 15 mM $MgCl_2$ and creatine phosphokinase activity as specified Reactions started by addition of 0 5 μmole 3-phosphoglycerate

considerable inhibition, whereas AMP at up to 4 mM had a comparatively small effect The sensitivity of this reaction to ADP is further illustrated in Fig 3, which shows the marked stimulatory effect of increasing creatine phosphokinase activity on the rate of NADPH oxidation in reaction mixtures containing 5 mM phosphocreatine The steady-state concentration of ADP in this case should be largely determined by the relative activities of phosphoglycerate kinase (forming ADP) and of creatine phosphokinase (phosphorylating ADP)

ADP has previously been reported as an inhibitor of purified spinach glyceraldehyde-3-phosphate dehydrogenase (assayed in the direction of NADP reduction) by Pupillo et al [13], although Muller [14] found no inhibitory effect of ADP In the present work, activation of this enzyme by ATP and NADPH [14, 15] during assays was avoided by illuminating the spinach leaves before isolating the chloroplasts and by preincubating reaction mixtures for 5 min prior to the addition of 3-phosphoglycerate

Stoichiometry of 3-phosphoglycerate-dependent NADPH oxidation by chloroplast extracts

The stoichiometry of 3-phosphoglycerate reduction was investigated by measuring the extent of NADPH oxidation consequent upon the addition of 0 1 μmole 3-phosphoglycerate to reaction mixtures (Fig 4) The amount of NADPH oxidised

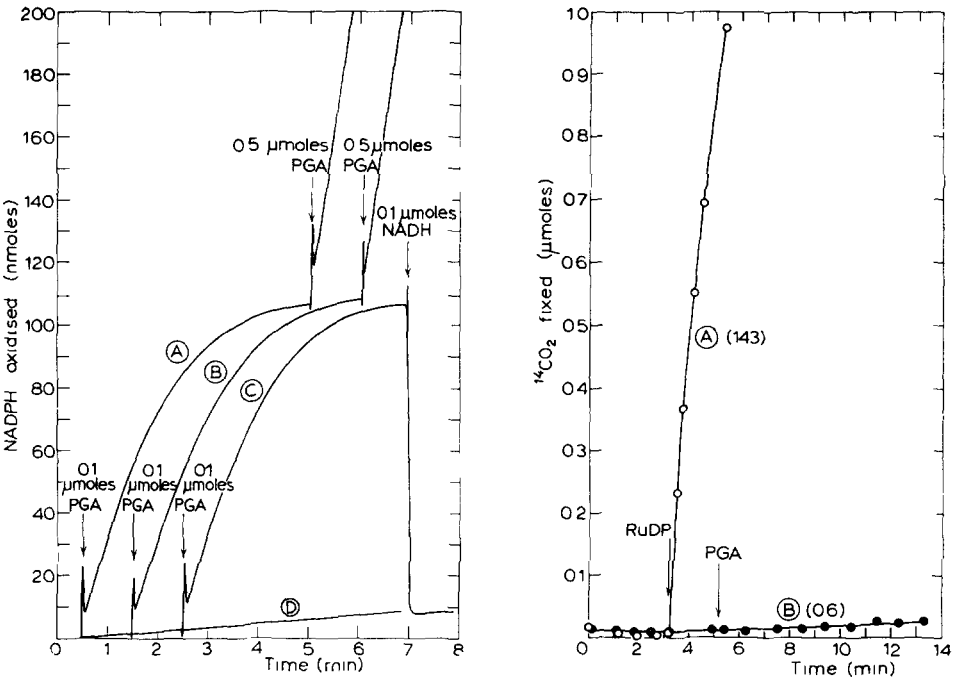


Fig 4 Recorder traces of 3-phosphoglycerate-dependent NADPH oxidation by chloroplast extract Basic reaction mixture (see Materials and Methods) plus 2 mM ATP, 15 mM $MgCl_2$, 5 mM phosphocreatine and 1 unit creatine phosphikinase activity In addition Expt B contained 10 mM $NaHCO_3$ Additions of 3-phosphoglycerate (PGA) and standard NADH where indicated Trace D, obtained in the absence of 3-phosphoglycerate is the base line for the other traces

Fig 5 Carbon fixation by reconstituted chloroplasts Basic reaction mixtures as for determination of oxygen evolution by reconstituted chloroplasts (see Materials and Methods) but including 100 μCi $NaH^{14}CO_3$ Determination of ^{14}C fixation by the method of Walker et al [16] Illumination of the reaction mixtures commenced at zero time, additions of 2 μmoles ribulose 1 5-diphosphate (RuDP) to Expt A and of 2 μmoles 3-phosphoglycerate (PGA) to Expt B where indicated The figures in brackets are the rates μmoles ^{14}C fixed/mg chlorophyll per h

was always found to be within 3 % of 0 1 μmole (trace A) With chloroplast extract and an ATP-regenerating system the possibility existed that the entire Benson–Calvin cycle might begin to turn over and convert newly produced triose phosphate to 3-phosphoglycerate via the carboxylation step However, no increase in the amount of NADPH oxidised was observed in the presence of 10 mM $NaHCO_3$ (Fig 4, trace B), and the rates of NADPH oxidation following the addition of a further 0 5 μmole 3-phosphoglycerate were equal in the presence and absence of 10 mM bicarbonate The low basal rate of NADPH oxidation in the absence of added 3-phosphoglycerate is shown in Fig 4, trace D Trace C is a calibration check with standard NADH

The absence of any appreciable Benson–Calvin cycle turnover in chloroplast extracts was confirmed by measuring 3-phosphoglycerate-dependent carbon fixation in the reconstituted chloroplast system containing 10 mM $NaH^{14}CO_3$ (Fig 5, B) The rate of carbon fixation was then less than 0 5 % of that which followed the addition of 0 5 mM ribulose 1,5-diphosphate (Fig 5, A)

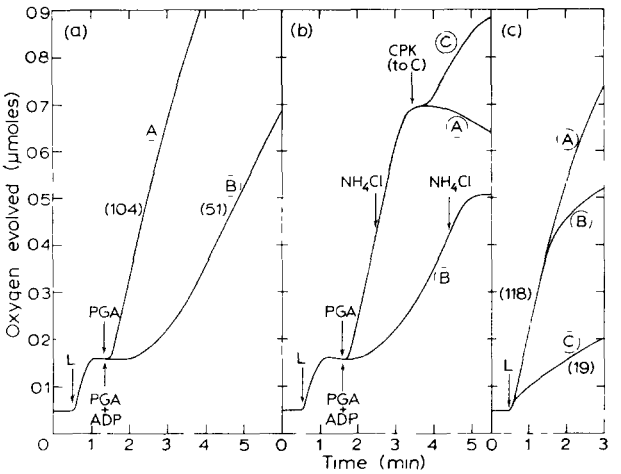


Fig 6 Recorder traces of photosynthetic O_2 evolution by reconstituted chloroplasts (a) Basic reaction mixture (see Materials and Methods), with additions of 2 μmoles 3-phosphoglycerate (PGA) and 1 μmole ADP where indicated Illumination commenced at L (b) As for (a) except that Expt C contained in addition of 5 mM phosphocreatine Additions of 5 μmoles NH_4Cl and of 2 units creatine phosphokinase activity (CPK) where indicated (c) As for (a) but with 1 mM NADP and with chloroplast extract omitted In addition, A contained 2 5 mM NH_4Cl with ATP omitted and B contained 0 5 mM ADP The figures in brackets are the rates μmoles O_2 evolved/mg chlorophyll per h

3-Phosphoglycerate-dependent oxygen evolution by reconstituted chloroplasts

On illumination of a reconstituted chloroplast reaction mixture, there was an initial rapid evolution of O_2 which ceased when the NADP present was reduced (Fig 6a) The addition of 3-phosphoglycerate then restarted O_2 evolution almost immediately (trace A) However, when ADP (0 5 mM) and 3-phosphoglycerate were added together (B), the rate eventually obtained was about half than in (A) In the absence of added ADP, the addition of the uncoupler, NH_4Cl initially caused a slight simulation of 3-phosphoglycerate-dependent O_2 evolution (Fig 6b, trace A), followed by complete inhibition as previously observed [2] In the presence of 5 mM phosphocreatine, this inhibition was reversed by the addition of creatine phosphokinase (trace C) The onset of inhibition following NH_4Cl addition was much more rapid in the reaction mixture which initially contained 0 5 mM ADP (trace B) Oxygen evolution from envelope-free chloroplasts illuminated in the presence of ferredoxin and substrate amounts of NADP (Fig 6c) was slow with ATP (trace C) and fast when uncoupled with NH_4Cl (trace A) With 2 mM ATP plus 0 5 mM ADP (trace B), the rate of O_2 evolution initially equalled that of uncoupled chloroplasts, then decreased as the ADP was phosphorylated

From these results it can be concluded that the inhibition of O_2 evolution from 3-phosphoglycerate by addition of ADP or of NH_4Cl was due to the inhibitory effect of ADP on the enzymic reduction of 3-phosphoglycerate demonstrated above The rather complex kinetics seen in Fig 6b are the result of the opposing effects of ADP on coupled NADP photo-reduction and on 3-phosphoglycerate-dependent NADPH oxidation The initial acceleration of O_2 evolution on addition of NH_4Cl

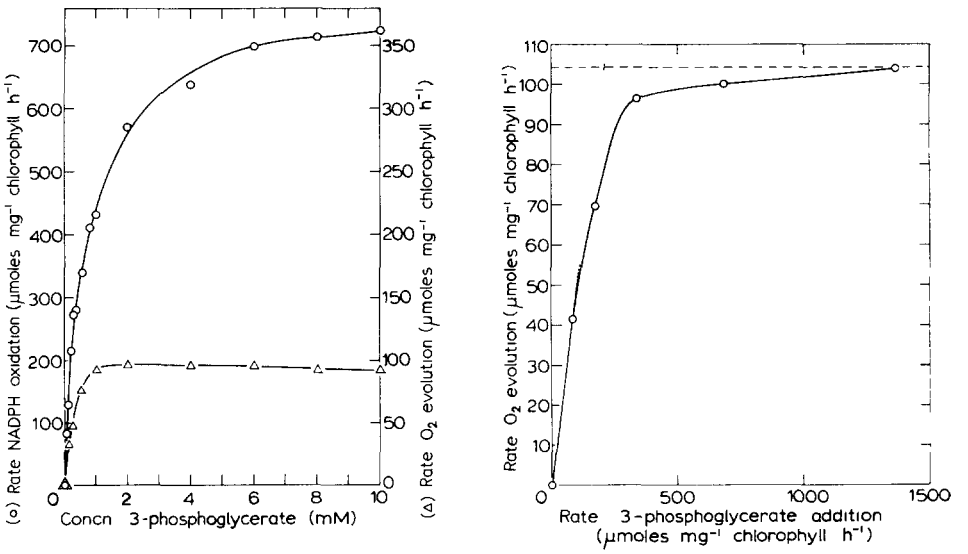


Fig 7 The effect of increasing 3-phosphoglycerate concentration on the rates of NADPH oxidation by chloroplast extract (circles) and O_2 evolution by reconstituted chloroplasts (triangles) Measurements of NADPH oxidation were made in basic reaction mixture (see Materials and Methods) plus 5 mM phosphocreatine and 1 unit creatine phosphokinase activity Reconstituted chloroplast reaction mixture as described in Materials and Methods

Fig 8 The effect of continuous addition of 3-phosphoglycerate at increasing rates on the rate of oxygen evolution by reconstituted chloroplasts The reaction mixture was as described in Materials and Methods After commencing illumination 3-phosphoglycerate was added continuously by a Radiometer ABU 12 variable speed titrator with an 0 25-ml syringe containing 40 mM 3-phosphoglycerate The dotted line represents the theoretical relationship of 1 mole O_2 evolved per 2 moles 3-phosphoglycerate added The horizontal dashed line represents the maximum rate of oxygen evolution observed by intact chloroplasts from the same preparation, measured in a reaction mixture containing 0 33 M sorbitol, 2 mM EDTA 1 mM $MgCl_2$ 1 mM $MnCl_2$, 50 mM *N*-2-hydroxyethylpiperzine-*N'*-ethanesulphonic acid, 10 mM $NaHCO_3$ 5 mM PP_i and 0 5 mM P_i pH 7 6

(Fig 6b, trace A) is attributed to NADP reduction under uncoupled conditions since the steady-state NADP pool should be largely oxidised when apparent glyceraldehyde-3-phosphate dehydrogenase activity is high In reaction mixtures containing added ADP the apparent dehydrogenase activity is low (Fig 6b, trace B), the NADP pool is largely reduced, and less O_2 is evolved after addition of NH_4Cl

The effect of 3-phosphoglycerate concentration on O_2 evolution by reconstituted chloroplasts is compared with its effect on NADPH oxidation by chloroplast extract in Fig 7 (assuming a stoichiometry of 2 moles NADPH oxidised/1 mole O_2 evolved) The rate of NADPH oxidation approached saturation at 10 mM 3-phosphoglycerate, whereas the rate of O_2 evolution was much lower and reached a ceiling just below 100 μmoles O_2/mg chlorophyll per h as it approached the rate of NADP-dependent oxygen evolution (118 μmoles O_2/mg chlorophyll per h) exhibited by uncoupled chloroplasts (Fig 6c, trace A) The limiting factor for 3-phosphoglycerate-dependent oxygen evolution therefore appears to be the rate of NADP reduction and the ability of photophosphorylation to maintain the steady-state concentration of ADP at a level sufficiently low to allow an adequate rate of 3-phosphoglycerate reduction

The progress of carboxylation in the reconstituted system was also simulated by continuously adding 3-phosphoglycerate, at various rates, from an automatic titrator (Fig 8) Added slowly, 2 moles of 3-phosphoglycerate gave rise to 1 mole O_2 (dotted line), but at higher rates the ratio O_2/3-phosphoglycerate declined rapidly as the O_2 evolution rate approached saturation The entire experimental curve conforms approximately to the theoretical boundaries described by the evolution of 1 mole O_2/2 moles 3-phosphoglycerate added and by a ceiling representing the rate of oxygen evolution (in the presence of saturating bicarbonate and 3-phosphoglycerate), by the intact chloroplasts from which the reconstituted system was prepared It therefore seems likely that the previously discussed factors, which limit the rate of 3-phosphoglycerate-dependent oxygen evolution by reconstituted chloroplasts, apply also to intact isolated chloroplasts

CONCLUSIONS

The rate of ribulose 1,5-diphosphate-dependent CO_2 fixation in the reconstituted chloroplast system (typically 140 μmoles $^{14}CO_2$ fixed/mg chlorophyll per h, Fig 5, A) can exceed the rate of 3-phosphoglycerate-dependent oxygen evolution (typically 100 μmoles O_2/mg chlorophyll per h, Fig 6a), because of the limitations imposed on the rate of 3-phosphoglycerate reduction by the steady-state ADP concentration and the availability of NADPH These factors probably also impose a limitation on the rate of photosynthesis by intact isolated chloroplasts

The negligible turnover of the Benson–Calvin cycle in the reconstituted chloroplast system used here is attributed to dilution of the stromal contents following osmotic shock of the chloroplast envelopes The specific space of the stroma compartment of spinach chloroplasts has recently been measured by Heldt et al [17] as 23 μl/mg chlorophyll Although the ratio of chlorophyll to stromal protein in the reconstituted system is the same as in the isolated intact chloroplasts, the reconstituted system is 400 times more dilute on the basis of Heldt's figure, and the concentration of those cycle intermediates normally contained within the chloroplast envelope will be reduced by a similar factor Turnover of the cycle has, however, been recently demonstrated [18, 19], in reconstituted chloroplast systems containing a large excess of stromal protein

The sensitivity of the rate of enzymic reduction of 3-phosphoglycerate to changes in ADP concentration suggest that the level of ADP in the intact chloroplast may be an important factor in the control of photosynthesis The decrease in chloroplast ADP concentration upon illumination [20] may also contribute to the reported light activation [15] of this reaction

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