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THE CONTROL OF 3-PHOSPHOGLYCERATE REDUCTION IN ISOLATED CHLOROPLASTS BY THE CONCENTRATIONS OF ATP, ADP AND 3-PHOSPHOGLYCERATE

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Summary

1. Oxygen evolution by reconstituted chloroplasts with 3-phosphoglycerate as substrate was inhibited by ADP. This inhibition was overcome by increased concentrations of 3-phosphoglycerate or by the additon of excess ATP.

2. The initial rate of 3-phosphoglycerate reduction by chloroplast stromal extracts, measured as 3-phosphoglycerate-dependent oxidation of NADPH, was also dependent on the concentrations of 3-phosphoglycerate, ADP and ATP.

3. Within the range of concentrations of 3-phosphoglycerate, ADP and ATP expected to occur within intact chloroplasts, the rate of 3-phosphoglycerate (PGA) reduction by stromal extract was proportional to the ratio [ATP] [PGA]/[ADP].

4. These results are consistent with the notion that the substrates and products of 3-phosphoglycerate kinase control the rate of 3-phosphoglycerate reduction via a mass action effect.

Introduction

In the intact chloroplast, the reduction of 3-phosphoglycerate to triose phosphate utilizes ATP and NADPH and so regenerates the ADP, NADP and P_i required for continued electron transport (Eqns. 1 and 2):

3-phosphoglycerate + ATP $\rightleftharpoons$ 1,3-diphosphoglycerate + ADP (1)

1,3-diphosphoglycerate + NADPH $\rightleftharpoons$ triose phosphate + NADP + P_i (2)

The reduction of 3-phosphoglycerate by reconstituted chloroplasts (thylakoids plus stromal protein) is inhibited by ADP [1]. The addition of relatively

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

low concentrations of ADP produced a lag period before oxygen evolution commenced and depressed the final rate. Similar effects have been observed in the reconstituted chloroplast system with substrates (such as ribose 5-phosphate and fructose 1,6-bisphosphate) which bring about the utilisation of ATP more rapidly than it is formed by photophosphorylation [2,3]. The reduction of glycerate 1,3-bisphosphate by reconstituted chloroplasts (Eqn. 2) is not affected by ADP [4] and the inhibition of 3-phosphoglycerate reduction by ADP has been attributed to increased conversion of glycerate 1,3-bisphosphate to 3-phosphoglycerate by phosphoglycerate kinase. This would be consistent with reports [5—7] that chloroplast phosphoglycerate kinase is inhibited by ADP when assayed in the direction of glycerate 1,3-bisphosphate formation.

If the inhibition of 3-phosphoglycerate reduction by ADP is the result of a mass action effect on phosphoglycerate kinase [4,8], it should be possible to reverse the inhibition by increased concentrations of the two substrates (3-phosphoglycerate and ATP). In view of the low values of ATP/ADP ratios measured in actively photosynthesizing chloroplasts [9,10] such control of phosphoglycerate kinase may be important in regulating CO_2 fixation. Our present results show that 3-phosphoglycerate and ATP can reverse the ADP inhibition of 3-phosphoglycerate reduction and that the rate of 3-phosphoglycerate reduction by reconstituted chloroplasts is proportional to the ratio [PGA] [ATP]/[ADP] within physiologically significant concentrations of these metabolites.

Materials and Methods

Spinach (*Spinacia oleracea*, United States Hybrid 424, Ferry Morse Seed Co., Mountain View, Calif.) was grown in water culture according to Lilley and Walker [1]. Peas (*Pisum sativum*, Feltham First, Sutton Seeds Reading, U.K.) were grown in vermiculite for 11—14 days in a glasshouse.

Intact chloroplasts were isolated as before [1]. For spinach chloroplasts, the isolation medium was 330 mM sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 2 mM sodium isoascorbate adjusted to pH 6.5 with HCl. The isolation medium for pea chloroplasts was 330 mM glucose, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 17 mM NaCl, 2 mM sodium isoascorbate, 0.1% (w/v) bovine serum albumin adjusted to pH 6.5 with HCl. The chloroplast resuspension medium was 330 mM sorbitol. 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer adjusted to pH 7.6 with KOH.

For the preparation of chloroplast extract, the pellet of intact chloroplasts was resuspended in 1 : 25 dilution of the above resuspension medium plus 3 mM dithiothreitol. This suspension of ruptured chloroplasts plus stromal protein was centrifuged at 15 000 × *g* for 10 min yielding a supernatant (chloroplast extract) which was free of chlorophyll. The pellet (envelope-free chloroplasts) was resuspended in full strength medium as for intact chloroplasts. The volume of chloroplast extract per mg chlorophyll was determined from the total volume of extract and the total chlorophyll content of the envelope-free chloroplasts and was corrected for the percentage of intact chloroplasts in the original chloroplast pellet.

Oxygen evolution was measured as described by Delieu and Walker [11]

using electrodes purchased from Hansatech Ltd. (King's Lynn, U.K.). The standard reaction mixture for measurement of 3-phosphoglycerate-dependent oxygen evolution by reconstituted chloroplasts contained envelope-free chloroplasts (100 μg chlorophyll) plus chloroplast extract (equivalent to 100 μg chlorophyll), 300 mM sorbitol, 10 mM KCl, 1 mM EDTA, 50 mM HEPES, 10 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM sodium isoascorbate, 2 mM P_i, 0.2 mM NADP, 0.4 mM ADP, 180 μg spinach ferredoxin and 220 units/ml catalase in 1 ml final volume at pH 8.0 and 20°C.

Reduction of 3-phosphoglycerate by chloroplast extracts in the dark was measured as the rate of oxidation of exogenous NADPH. The standard reaction mixture was the same as for the measurement of oxygen evolution except that NADP, ADP and ferredoxin were replaced by 0.25 mM NADPH plus 1.5 mM ATP. Envelope free chloroplasts were omitted and the amount of chloroplast extract was equivalent to approx. 5 μg chlorophyll (70—90 μg protein; total volume 1 ml; path length 10 mm). The reaction was started by the addition of 3-phosphoglycerate and changes in absorbance at 340 nm was measured at 20°C with a Gilford spectrophotometer.

Fresh solutions of NADPH, creatine phosphate and creatine phosphokinase were prepared each day from desiccated solids. Spinach ferredoxin was prepared by the method of Rao et al. [12]. Chlorophyll was determined by the method of Arnon [13] and protein by the biuret method [14]. The percentage of intact chloroplasts was measured by the ferricyanide method [15].

Results

In the reconstituted chloroplast system, the addition of ADP produces a lag period before 3-phosphoglycerate reduction commences and the final rate of oxygen evolution is depressed [1]. If this inhibition results from a mass action effect on 3-phosphoglycerate kinase, addition of excess 3-phosphoglycerate and ATP should prevent the inhibitory effect of ADP. Recorder traces of 3-phosphoglycerate-dependent oxygen evolution by reconstituted chloroplasts are shown in Figs. 1 and 2. In both cases, additional 1 mM ADP was added after all the NADP had been reduced. Following subsequent additions of low concentrations of 3-phosphoglycerate (Figs. 1B and 2B) there was a marked lag period before oxygen evolution commenced, reflecting the time taken for the chloroplasts to phosphorylate sufficient of the added ADP to reduce its inhibitory effect on 3-phosphoglycerate kinase [1,4]. This lag was largely prevented by the addition of higher concentrations of 3-phosphoglycerate (Fig. 1A) or by the addition of excess ATP (Fig. 2A). Both ATP and higher concentrations of 3-phosphoglycerate also gave increased rates of oxygen evolution. The control experiment (no additional ADP, low concentrations of 3-phosphoglycerate), closely resembled the traces obtained with excess ATP or 3-phosphoglycerate. Addition of ATP, or higher concentrations of 3-phosphoglycerate after oxygen evolution had commenced resulted in a rapid stimulation of oxygen evolution (Figs. 1B and 2B).

These experiments show that the inhibition of 3-phosphoglycerate reduction by ADP can be reversed if the concentrations of ATP or 3-phosphoglycerate are sufficiently increased. This is in agreement with earlier suggestions that chloro-

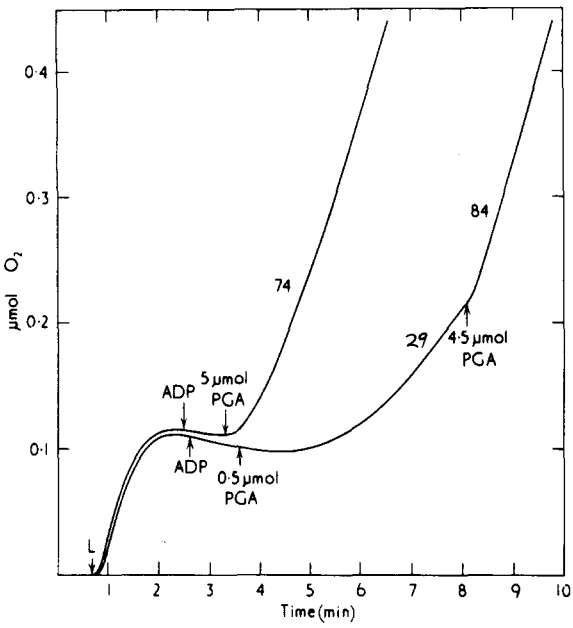

Fig. 1. Recorder traces of oxygen evolution by reconstituted pea chloroplasts showing reversal of ADP inhibition by increased concentrations of 3-phosphoglycerate. After all the NADP was reduced, 1 µmol ADP was added to both mixtures as indicated and the oxygen evolution was started by the addition of 3-phosphoglycerate (0.5 or 5 µmol). Where indicated, a further 4.5 µmol 3-phosphoglycerate was added to the lower trace. Numbers alongside the traces indicate rates of oxygen evolution in µmol O_2/mg chlorophyll per h. PGA, 3-phosphoglycerate.

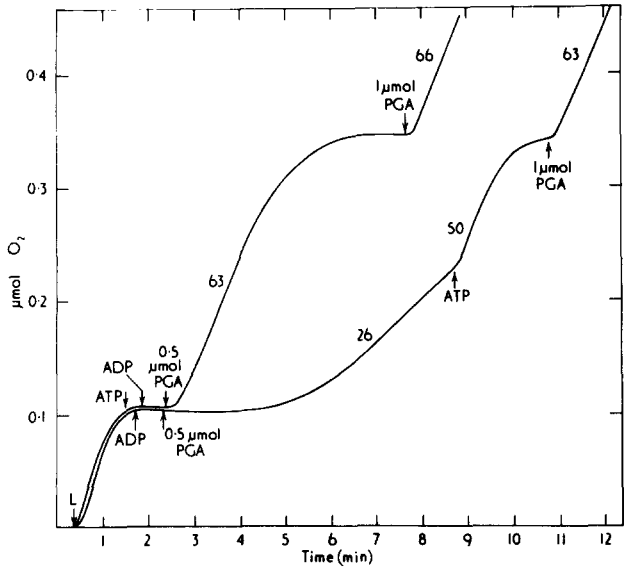

Fig. 2. Recorder traces of oxygen evolution by reconstituted pea chloroplasts showing reversal of ADP inhibition by ATP. Conditions were the same as for Fig. 1. Additions, where indicated, were 1 µmol ADP, 5 µmol ATP and 0.5 µmol 3-phosphoglycerate (PGA). After all the 3-phosphoglycerate had been reduced, a further 1 µmol was added to both mixtures.

plast 3-phosphoglycerate kinase is controlled by mass action effects [8]. Furthermore, it appears that the rate of 3-phosphoglycerate reduction by reconstituted chloroplasts is largely determined by the concentrations of ATP, ADP and 3-phosphoglycerate (in the presence of NADPH and glyceraldehyde-3-phosphate dehydrogenase, the other product of 3-phosphoglycerate kinase, 1,3-diphosphoglycerate, would be reduced to triose phosphate and the steady state concentration of 1,3-diphosphoglycerate would probably be kept low). It was therefore of interest to study the effect of different concentrations of these metabolites on the rate of 3-phosphoglycerate reduction. The recent work of Lilley et al. [10] suggests that the concentration of total adenine nucleotides in intact chloroplasts is 1.0—1.5 mM with an ATP/ADP ratio varying from 1.5 during induction to 0.2—0.3 during steady state photosynthesis. Their results also suggest that the concentration of 3-phosphoglycerate in intact chloroplasts can be as high as 3—4 mM during steady state photosynthesis. (It should be noted that measurements of stromal metabolite concentrations give average values and do not allow for the possibility of concentration gradients within the chloroplast. Thus a difference in the ratio ATP/ADP might be expected between the thylakoid surface, where ATP is produced, and the chloroplast stroma where it is consumed).

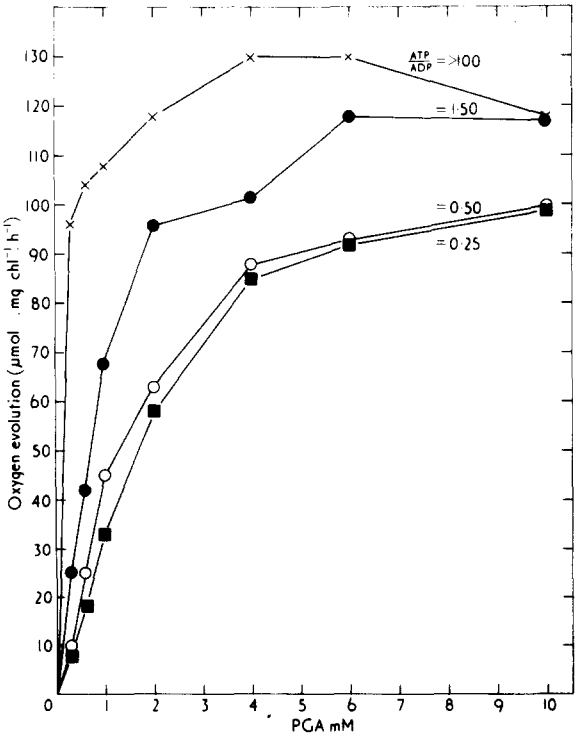

Fig. 3. Effect of increasing concentrations of 3-phosphoglycerate on the rate of oxygen evolution of reconstituted pea chloroplasts. ADP was initially omitted. After all the NADP had been reduced, ADP, ATP and 3-phosphoglycerate were added together to give the concentrations of 3-phosphoglycerate and ratios of ATP/ADP shown. The concentration of total adenine nucleotides was kept constant at 1.5 mM. The maximum rate of oxygen evolution reached is plotted.

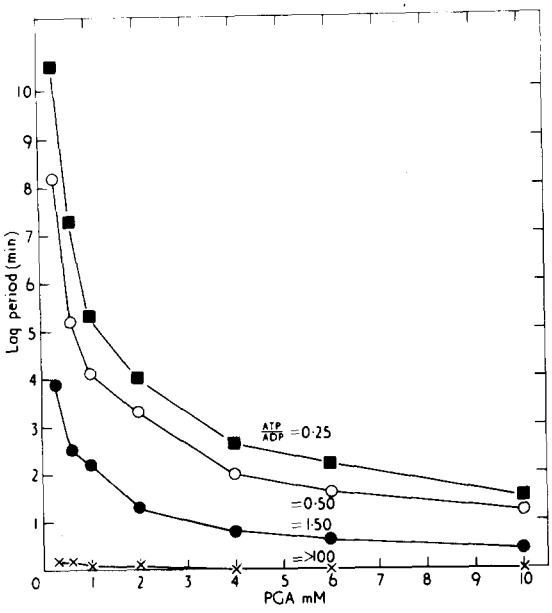

Fig. 4. Effect of increasing concentrations of 3-phosphoglycerate on the time taken to reach maximum rate of oxygen evolution by reconstituted pea chloroplasts. The data were obtained from the experiment described in Fig. 3.

Figs. 3 and 4 show the results of experiments designed to mimic the range of conditions expected to occur in the intact chloroplast. The concentration of adenine nucleotides was kept constant at 1.5 mM whilst the ATP/ADP ratio varied from greater than 100 (no added ADP) to 0.25. For the experiments of

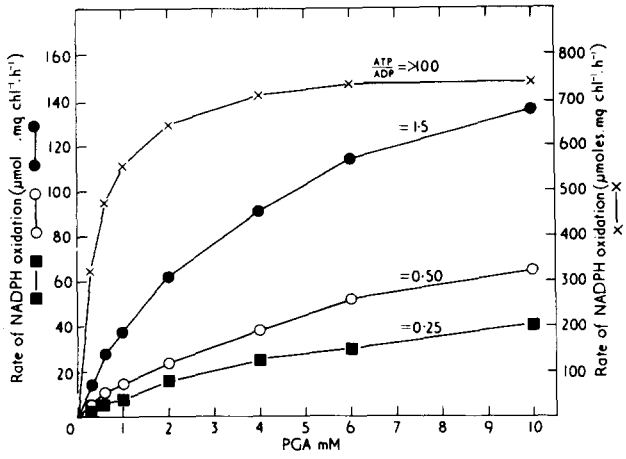

Fig. 5. Effect of increasing concentrations of 3-phosphoglycerate on the rate of NADPH oxidation by chloroplast extracts. ATP was initially omitted and chloroplast extract containing 86 μg protein was added. The reaction was started by the simultaneous addition of 3-phosphoglycerate, ADP and ATP as described in Fig. 4. The final volume was 1.0 ml. The top curve (ATP/ADP > 100) also contained 10 mM creatine phosphate and 4 units creatine phosphokinase and is plotted on a different scale to the other curves. The rate of NADPH oxidation in the first 30 s is plotted.

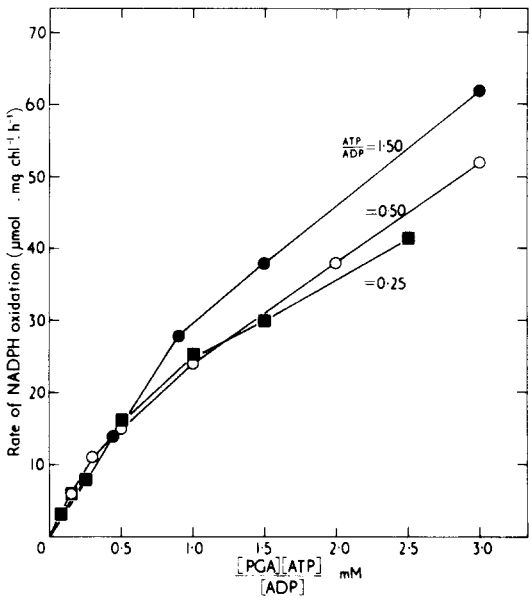

Fig. 6. Effect of the ratio [PGA] [ATP]/[ADP] on the rate of NADPH oxidation by chloroplast extracts. The data are from Fig. 5.

Figs. 3 and 4, ADP was omitted from the reconstituted chloroplast systems and appropriate amounts of ATP, ADP and 3-phosphoglycerate were added together (after all the added NADP was reduced) to produce the conditions described. When all the adenine nucleotides were added as ATP (Fig. 3, top curve) the rate of 3-phosphoglycerate-dependent oxygen evolution was saturated at 2—3 mM 3-phosphoglycerate. This is in agreement with previous results [1] and represents the ceiling imposed by the rate of electron transport. As the ATP/ADP ratio was decreased, the rate of oxygen evolution was decreased and higher concentrations of 3-phosphoglycerate were required to achieve a given rate of oxygen evolution (Fig. 3). In all cases where ADP was added, there was a lag period before the maximum rate of oxygen evolution was achieved similar to that shown in Figs. 1 and 2. The lag period for these experiments is plotted as a function of 3-phosphoglycerate concentration in Fig. 4. When no ADP was added, the lag was extremely short at all 3-phosphoglycerate concentrations used. As the ATP/ADP ratio was decreased, longer lags were observed, particularly at low 3-phosphoglycerate concentrations. From Figs. 3 and 4 it is apparent that both the lag and the maximum rate were dependent on the concentration of 3-phosphoglycerate and on the ATP/ADP ratio.

In the reconstituted chloroplast system, the concentrations of metabolites are continually altered by the system. Thus given sufficient time the chloroplasts could phosphorylate all the added ADP and the rate would become independent of the initial ATP/ADP ratio. In addition, the concentration of 3-phosphoglycerate will be continually decreased as it is reduced by the chloroplasts. For this reason, the initial rate of 3-phosphoglycerate reduction by chloroplast extracts (stromal protein) was measured directly as the rate of NADPH oxidation. Where all the adenine nucleotides were added as ATP,

creatine phosphate and creatine phosphokinase were also included to phosphorylate any ADP formed. In the presence of such an ATP regenerating system, relatively high rates of NADPH oxidation were observed and the activity was saturated at 6—10 mM 3-phosphoglycerate (Fig. 5, top curve). Decreasing the ATP/ADP ratio to 1.5 greatly decreased the rate of NADPH oxidation and the reaction was not saturated at 10 mM 3-phosphoglycerate (Fig. 5.) Further decreases in the ATP/ADP ratio also resulted in decreased rates of NADPH oxidation. It is interesting to note that in all cases where ADP was included, the rates of NADPH oxidation by the chloroplast extracts were lower than those expected from the rates of 3-phosphoglycerate-dependent oxygen evolution by the reconstituted system, assuming 2 mol NADPH oxidized per mol O_2 evolved (cf. Figs. 3 and 5).

The data from Fig. 5 are replotted in Fig. 6 as a function of the ratio [PGA] [ATP]/[ADP]. The three curves obtained for different ATP/ADP ratios are very similar when plotted in this manner, particularly at values of the ratio less than 1 mM (Fig. 6). Using the data obtained by Heldt et al. [10,16], the value of this ratio in intact chloroplasts during CO_2 fixation varies between 0.5 and 1.7 mM.

Discussion

Although the overall reduction of 3-phosphoglycerate to triose phosphate is freely reversible, the initial formation of glycerate 1,3-bisphosphate from 3-phosphoglycerate has an unfavourable $\Delta F'$ (about 4.5 kcal) which, as a consequence of mass action, makes this stage particularly susceptible to inhibition by ADP. Data presented in this paper indicates that the ADP inhibition is diminished by increased 3-phosphoglycerate or ATP in accord with this thermodynamic evaluation. It has also been proposed [5—7] that the enzymic catalysis of this reaction is governed in an allosteric manner by energy charge [17]. It should be noted, however, that the observed responses to energy charge do not in themselves permit the conclusion that the control is of an allosteric nature nor do they eliminate effects such as end-product inhibition or mass action. In any event the final effect will be much the same whatever the mechanism involved. What we therefore believe to be much more important about the present results is the demonstration that the rate of 3-phosphoglycerate reduction in chloroplast extracts is markedly affected by [PGA] [ATP]/[ADP] when the relative concentrations of these compounds are manipulated within the range which might reasonably be expected to occur inside the chloroplast. It follows that there could be no significant reduction of 3-phosphoglycerate in any circumstances in which ATP consumption in other reactions approached the same rate as the regeneration of ATP by photophosphorylation. Moreover, it seems likely that 3-phosphoglycerate reduction will only proceed at its maximal rate in the presence of a large 3-phosphoglycerate pool within the illuminated stroma. Both of these conclusions are borne out by measurements which show that stromal 3-phosphoglycerate is lower during induction and increases to a high value in the steady state, whereas pentose monophosphates (a potential ATP sink via the phosphoribulokinase reaction) remain low [10]. Similarly there is ample evidence from the reconstituted

system [2,3,8] that 3-phosphoglycerate reduction is inhibited by ribose 5-phosphate or any other system which constitutes a potential ATP sink (such as glucose plus hexokinase) and that these inhibitions may be reversed by increasing the rate of phosphorylation (e.g. by adding extra ferredoxin or an ATP generator such as creatine phosphate and its kinase).

The physiological consequence of these effects are considerable. We would suggest, for example, that when photosynthesis is slowed by low P_i, a response which has been held to be of considerable importance in the regulation of photosynthetic carbon assimilation [8,18], that the shortage of P_i will be 'perceived' within the stroma by a decline in the [ATP]/[ADP] ratio and a consequent slowing of 3-phosphoglycerate reduction and ribose bisphosphate regeneration [19]. Because the total phosphate within the chloroplast remains more or less constant [10] export of sugar phosphates and the like will be diminished and a high [PGA]/[P_i] ratio will favour allosteric activation of ADP glucose pyrophosphorylase [20] thereby deflecting more of the carbon traffic towards starch synthesis and away from utilisation in the transketolase reaction. The evidence that P_i greatly influences the rate of photosynthesis and the distribution of the products between export and internal starch synthesis is considerable [8,18] and derives from experiments with leaf tissues [21] as well as with isolated chloroplasts [22].

The reduction of 3-phosphoglycerate in chloroplasts will also be readily affected by the [ATP]/[ADP] ratio whenever this is increased or decreased by corresponding changes in light intensity.

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