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TRANSIENT INHIBITION BY RIBOSE 5-PHOSPHATE OF PHOTOSYNTHETIC O_2 EVOLUTION IN A RECONSTITUTED CHLOROPLAST SYSTEM

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SUMMARY

Photosynthetic oxygen evolution by a reconstituted chloroplast system utilising *sn*-phospho-3-glycerol (3-phosphoglycerate) ceases upon the addition of ribose 5-phosphate even though the presence of this metabolite permits a rapid and immediate CO_2 fixation. The period of cessation is appreciable at 0.1 mM ribose 5-phosphate. It is lengthened as the amount of added ribose 5-phosphate is increased and by the addition of dithiothreitol, a known activator of ribulose-5-phosphate kinase. Ribulose 1,5-bisphosphate is without effect. A similar interruption of O_2 evolution may also be brought about by the addition of ADP or by ADP-generating systems such as glucose plus hexokinase. Spectrophotometric experiments indicate that the reoxidation of NADPH in the presence of *sn*-phospho-3-glycerol is similarly affected.

The transient inhibition by ribose 5-phosphate is not observed in the presence of an active ATP-generating system or in the presence of sufficient DL-glyceraldehyde to inhibit ribulose-5-phosphate kinase activity.

It is concluded that ribose 5-phosphate inhibits photosynthetic O_2 evolution by adversely affecting the steady-state ATP/ADP ratio and consequently the reduction of *sn*-phospho-3-glycerol to glyceraldehyde 3-phosphate. The results are discussed in their relation to ADP regulation of photosynthetic carbon assimilation and metabolite transport.

INTRODUCTION

The reconstituted chloroplast system [1–3] (envelope-free chloroplasts plus stromal protein, catalytic ADP, NADP and cofactors such as Mg^{2+}) will evolve O_2 when illuminated in the presence of *sn*-phospho-3-glycerol [1]. Electrons are passed from water to NADP which is then reoxidised by the natural oxidant (*sn*-glycerol 1,3-bisphosphate) as it is reduced to glyceraldehyde 3-phosphate. The oxidant is formed from *sn*-phospho-3-glycerol at the expense of ATP generated by photophosphorylation.

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

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The overall reduction of *sn*-phospho-3-glycerol to glyceraldehyde 3-phosphate, and its associated O_2 evolution in the reconstituted system is strongly inhibited by ADP [4] and there is now clear evidence (Slabas and Walker, ref. 5) that it is the first of the two partial reactions (i.e. the reaction catalysed by *sn*-phospho-3-glycerol kinase) which is affected.

This paper reports a similar inhibition of *sn*-phospho-3-glycerol-dependent O_2 evolution by Rib-5-*P* and presents evidence that ADP formed during the phosphorylation of Rbu-5-*P* is again the causal agent.

EXPERIMENTAL

Materials. Spinach (*Spinacia oleracea* United States Hybrid 424, Ferry-Morse Seed Co., P.O. Box 100, Mountain View, Calif.) was grown in water culture according to Lilley and Walker [4]. Biochemicals and enzymes were obtained from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey. Ferredoxin was prepared as described by Rao et al. [6]. All remaining reagents were purchased from Fisons Ltd., Loughborough and were of the highest purity available.

Intact chloroplasts, chloroplast extract and thylakoids. Intact chloroplasts were prepared as before [4] in 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$ and 2 mM isoascorbate at pH 6.5 and resuspended in 0.33 M sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) adjusted to pH 7.6 with KOH. For chloroplast extract [4] the pellet of intact chloroplasts (approx. 3.0 mg chlorophyll) was resuspended in 8 ml 1/25 dilution resuspending medium and centrifuged for 10 min at 13 000 $\times$ *g*. The supernatant chloroplast extract was dialysed in a Sartorius membrane (against one-tenth dilution resuspending medium) under reduced pressure for 45 min at 0 °C, to a volume of approx. 2.5 ml.

Chlorophyll was measured according to Arnon [7].

NADPH oxidation was measured spectroscopically at 340 nm in a final reaction volume of 1 ml at 20 °C using a Pye Unicam SP 800 spectrophotometer and a Rikadenki recorder. The basic reaction mixture contained chloroplast extract $\equiv$ 100 μg chlorophyll, 330 mM sorbitol, 2 mM EDTA, 6 mM $MgCl_2$, 0.16 mM NADPH, 4 mM creatine phosphate, 10 mM $NaHCO_3$, and 50 mM HEPES at pH 7.6.

O_2 evolution was measured polarographically [4] using twin Hansatech electrodes (Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk) as described by Delieu and Walker [8]. All O_2 measurements were made at 20 °C and in a final volume of 1 ml unless otherwise stated. Reaction mixtures (pH 7.9) normally contained 330 mM sorbitol, 50 mM HEPES, 10 mM KCl, 1.0 mM EDTA, 0.1 mM $NADP^+$, 0.1 mM ADP, 2 mM K H_2PO_4, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 4 mM sodium isoascorbate and 110 units catalase, and 90 μg ferredoxin per ml. Envelope-free chloroplasts and chloroplast extract were normally used in quantities equivalent to 100 μg chlorophyll.

Illumination. Reaction mixtures were illuminated with light from 150 W quartz iodine slide projectors. The light was passed through 15 cm of water, a Balzar Calflex C interference filter and red perspex (I.C.I. 400) to give light in the wavelength range 590–750 nm at an irradiance of 300 $W \cdot m^{-2}$.

CO_2 fixation. Samples (10 μl) were withdrawn from reaction mixtures at

timed intervals and injected into 40 μl of 0.3 M HCl, 0.01 M sucrose for determination of acid-stable radioactivity. Aliquots (20 μl) were dried on lens tissue discs on planchettes and radioactivity measured in a gas-flow counter. Radioactivity was related to CO_2 fixed by reference to [^{14}C]sucrose standards counted under identical conditions.

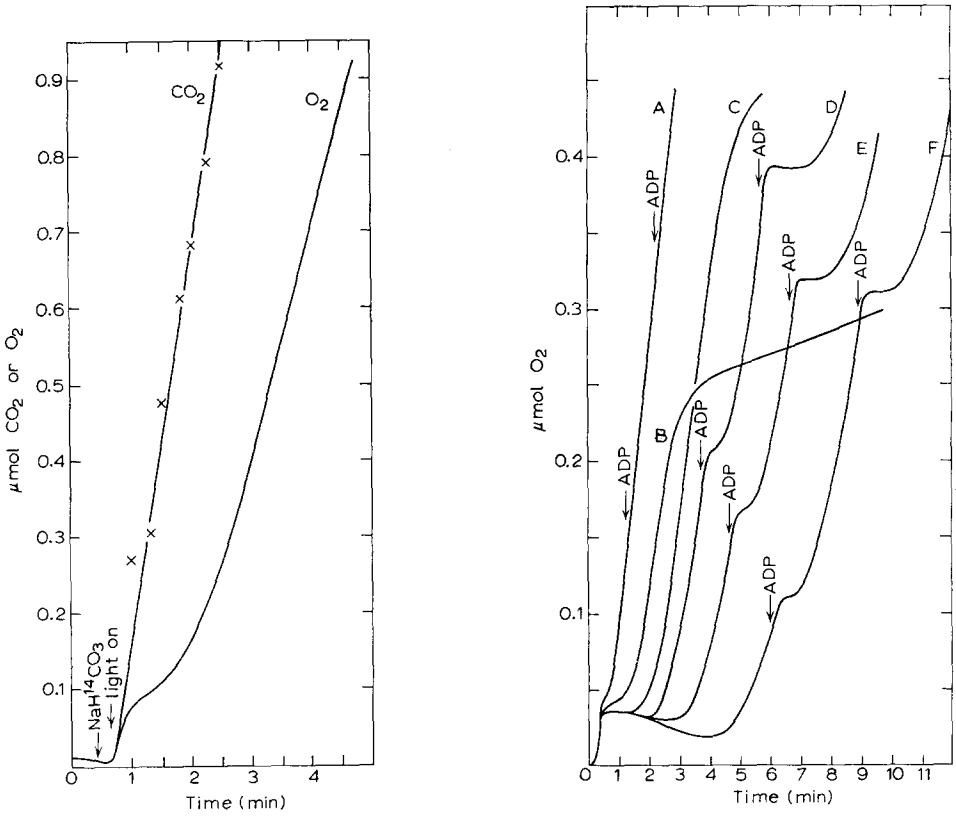


Fig. 1. Simultaneous measurement of photosynthetic CO_2 fixation and O_2 evolution in the reconstituted system. To show lag in O_2 evolution caused by Rib-5-*P* (2 mM) despite presence of *sn*-phospho-3-glycerol (2 mM). In this experiment the volume was increased to 2 ml and the reaction mixture also differed from the standard (see Experimental) in the following respects. Envelope-free chloroplasts, $\equiv$ 400 μg chlorophyll; chloroplast extract, $\equiv$ 800 μg chlorophyll; $NaH^{14}CO_3$, 20 mM (120 μCi); ferredoxin, 360 μg; ADP, 0.2 mM; NADP, 0.2 mM; K_2HPO_4, 4 mM; catalase, 220 units.

Fig. 2. Photosynthetic O_2 evolution by the reconstituted system to show increasing lag occasioned by increasing Rib-5-*P* concentration and the abolition of this lag by the inclusion of an additional ATP-generating system. Standard mixture (Experimental) with changes as follows. Envelope-free chloroplasts, $\equiv$ 200 μg chlorophyll; chloroplast extract, $\equiv$ 150 μg chlorophyll. In addition Rib-5-*P* was added in the following concentrations: A, 0.7 mM; B, 0.2 mM; C, 0,4 mM; D, 0.7 mM; E, 1.1 mM and F, 1.6 mM. Mixture A also contained 5 mM creatine phosphate and 4 units of creatine phosphate kinase. Subsequently ADP was added as indicated, 0.2 μmol at the first arrow and 0.6 μmol at the second.

RESULTS

Experiments with the reconstituted chloroplast system

In the absence of added substrate, the illuminated reconstituted system evolves O_2 until the NADP which is present is reduced. At this stage O_2 evolution may be immediately reinitiated by the addition of *sn*-phospho-3-glycerol [1, 3] and if *sn*-phospho-3-glycerol is present from the outset O_2 evolution continues (frequently at an unchanged rate) when it would otherwise cease for lack of oxidised NADP. Fig. 1 shows that if Rib-5-*P* is also present, only the O_2 evolution associated with the reduction of the catalytic NADP is at first observed (cf. Walker [9]) and there is then a lag before O_2 evolution is resumed. This lag is observed despite the fact that Rib-5-*P* promotes CO_2 fixation, that this fixation proceeds without appreciable delay (Fig. 1) and that *sn*-phospho-3-glycerol is the end-product of CO_2 fixation. Because CO_2 fixation and CO_2-dependent O_2 evolution involve the sequence of events illustrated in Fig. 10 and because of the known sensitivity of *sn*-phospho-3-glycerol reduction to ADP (Lilley and Walker [4]) it seemed reasonable to suppose that the lag observed in the presence of Rib-5-*P* [3, 7] might result from an increase in the steady-state concentration of ADP consequent upon the introduction of an ATP sink. This hypothesis allowed several predictions. If Rib-5-*P* is rapidly converted to Rbu-5-*P* (which drains ATP in the formation of Rbu-1,5-P_2) then the lag in CO_2-dependent O_2 evolution (Fig. 1) should also be seen with Rib-5-*P* alone (i.e. in the absence of added *sn*-phospho-3-glycerol). Moreover, the lag should increase with increasing Rib-

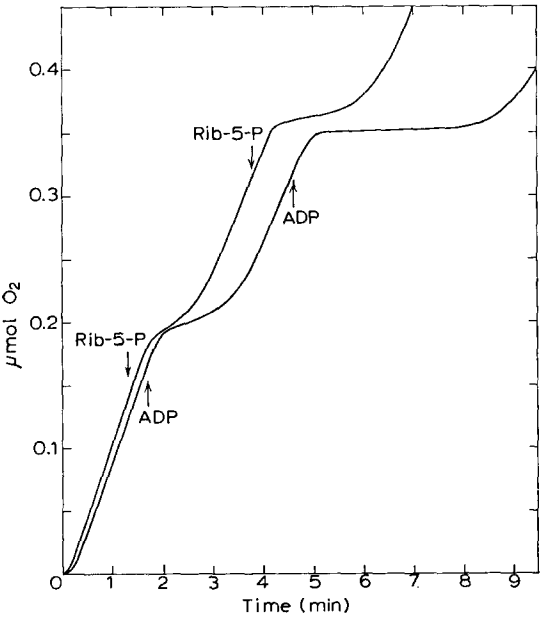


Fig. 3. Interruption of *sn*-phospho-3-glycerol-dependent O_2 evolution by Rib-5-*P* and ADP. The standard mixture (Experimental) also contained *sn*-phospho-3-glycerol (1 mM) and additional chloroplast extract (total ≡ 200 μg chlorophyll). Subsequently ADP and Rib-5-*P* were added as indicated, 0.2 μmol at the first arrow and 0.4 μmol at the second.

5-*P* and should be abolished in the presence of a sufficiently active ATP-regenerating system to overcome the ADP inhibition. Fig. 2 shows that all of these predictions were borne out. Similarly, there was no lag if Rbu-1,5-P_2 was substituted for Rib-5-*P*. Fig. 2 also shows that once steady-state O_2 evolution has been attained it may be interrupted by the addition of ADP. The interruption is more marked if the quantity of ADP is increased (Fig. 2) but there is no response in the presence of the artificial ATP generator (Fig. 2A) just as there is no appreciable response to Rib-5-*P* in the first instance (cf. Figs. 2A with 2C). (It may also be noted (Fig. 2B) that if the quantity of added Rib-5-*P* is sufficiently small (0.2 μmol, in this instance) rapid O_2 evolution falls off in accordance with the stoichiometry 1 Rib-5-*P* ≡ 2 *sn*-phospho-3-glycerol ≡ 1 O_2 and gives place to a slower rate which reflects the further metabolism of triose phosphate [10] in the reactions of the Benson-Calvin cycle.)

In Figs. 3–7 the ADP inhibition hypothesis is explored further. Fig. 3 shows that *sn*-phospho-3-glycerol-dependent O_2 evolution can be interrupted by the addition of Rib-5-*P* as well as by the addition of ADP (cf. Lilley and Walker [4]). Dithiothreitol, a known activator of phosphoribulokinase [11] accentuates the Rib-5-*P* effect (Fig. 4). In Fig. 5 an entirely different ATP sink (the familiar combination of glucose and hexokinase) is shown to interrupt *sn*-phospho-3-glycerol-dependent O_2 evolution in the same way as Rib-5-*P* and mannose and hexokinase acts in the same fashion. Fig. 6 shows that the Rib-5-*P* effect can be virtually eliminated in the

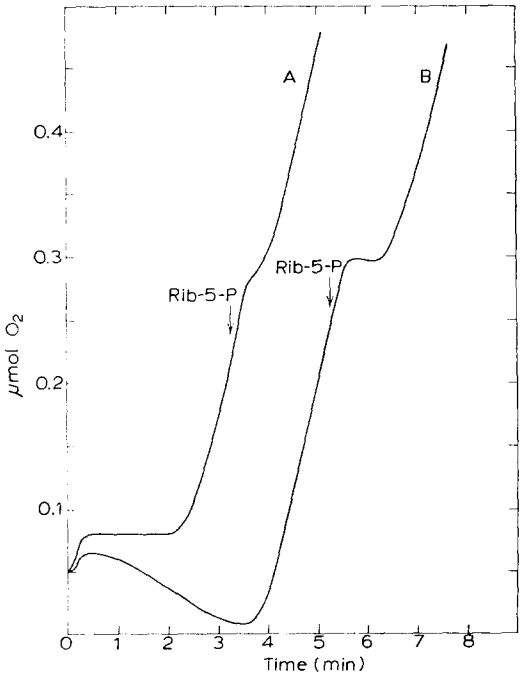
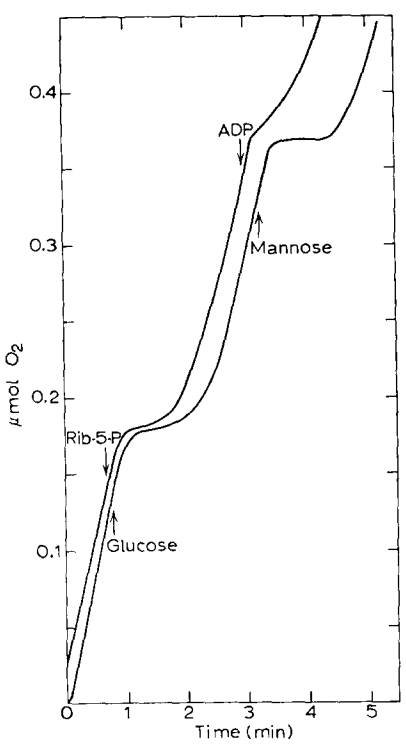


Fig. 4. Effect of dithiothreitol and Rib-5-*P* on *sn*-phospho-3-glycerol-dependent O_2 evolution in the reconstituted chloroplast system. The reaction mixture differed from the standard as follows: chloroplast extract, ≡ 150 μg chlorophyll; $MgCl_2$, 15 mM; and *sn*-phospho-3-glycerol, 1 mM. Reaction mixture B also contained 10 mM dithiothreitol. Rib-5-*P* (0.2 μmol) was added as indicated.



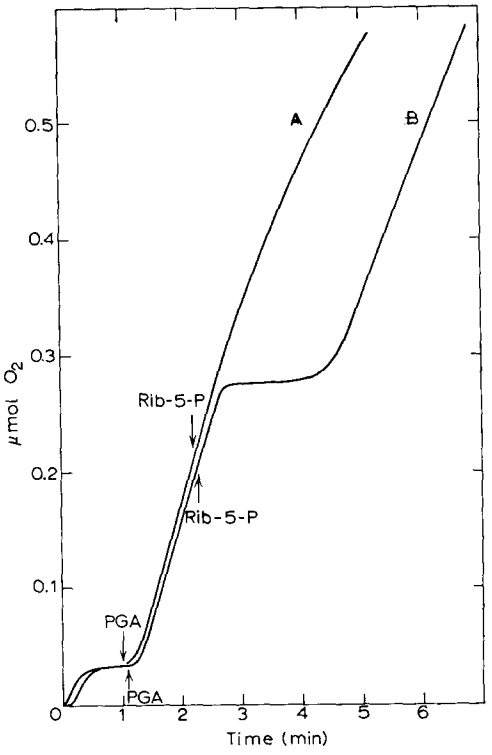


Fig. 5. Effect of Rib-5-*P*, ADP, mannose and glucose on *sn*-phospho-3-glycerol-dependent O_2 evolution in the reconstituted system. The reaction mixture differed from the standard as follows: chloroplast extract, ≡ 200 µg chlorophyll; $MgCl_2$, 15 mM; dithiothreitol, 5 mM; hexokinase, 10 units; and *sn*-phospho-3-glycerol, 1 mM. Subsequently Rib-5-*P* (0.2 µmol), glucose (0.2 µmol), mannose (0.2 µmol) and ADP (0.2 µmol) were added as indicated.

Fig. 6. Diminution, by DL-glyceraldehyde, of Rib-5-*P* interruption of *sn*-phospho-3-glycerol (PGA) dependent O_2 evolution. *sn*-phospho-3-glycerol (2 mM) and Rib-5-*P* (0.5 mM) added as indicated. Mixture A also contained 20 mM DL-glyceraldehyde.

presence of 20 mM DL-glyceraldehyde. In other experiments, not illustrated, 10 mM DL-glyceraldehyde was less effective in countering the Rib-5-*P* response (diminishing the lag by 50 %) but in the presence of 20 mM DL-glyceraldehyde there was no immediate discernible effect on O_2 evolution even when the Rib-5-*P* was raised to 0.75 mM and the consequent interruption in the control was extended to approx. 3 min. Phosphoribulokinase is known [12] to be inhibited by DL-glyceraldehyde (K_i = 20 mM) and these results are therefore a clear indication that the Rib-5-*P* effect is related to its conversion to Rbu-1,5-P_2 and its consequent function as an ATP sink (or ADP generator).

In general, the rates of O_2 evolution and CO_2 fixation observed (Figs. 1–7) are consistent with the conclusion that the transient inhibitions by Rib-5-*P* cease when most or all of this substrate is converted to Rbu-5-*P* and *sn*-phospo-3-glycerol in the appropriate partial reactions of the Benson-Calvin cycle (Fig. 10).

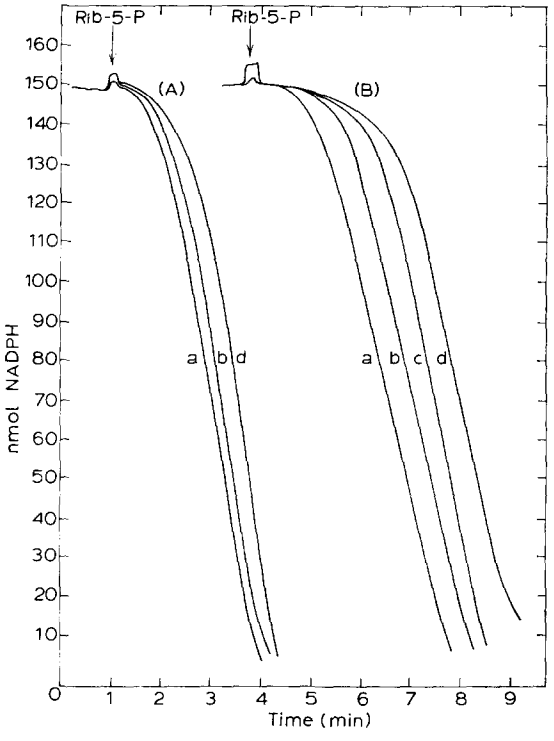


Fig. 7. Spectrophotometric measurements of NADPH oxidation by chloroplast extract with *sn*-phospho-3-glycerol as substrate. To show increasing lag occasioned by increasing Rib-5-*P* concentration and the abolition of this lag by the inclusion of an additional ATP-generating system. Reaction mixtures as described in Experimental plus 0.1 mM ATP. Series A contained 0.8 unit creatine phosphokinase and Series B 4 units creatine phosphokinase. The Rib-5-*P* concentrations were: a, 0.25 mM; b, 0.5 mM; c, 0.75 mM and d, 1.0 mM.

Spectrophotometric experiments

In the reconstituted system oxygen evolution reflects the rate of NADP reduction by water and in the presence of catalytic NADP this is directly related to the rate of reoxidation of NADPH. Reoxidation of NADPH may also be measured spectrophotometrically in a simplified system from which the envelope-free chloroplasts are omitted and in which catalysis depends upon the presence of stromal protein (chloroplast extract). In the absence of illuminated thylakoids. ATP must be supplied as such or by the inclusion of an ATP generator. Fig. 7 shows that in mixtures of this sort there is an initial lag in NADPH oxidation (cf. the lag in O_2 evolution in Fig. 2) and that the lag is greatest when the Rib-5-*P* concentration is highest. Moreover, the lag may be shortened by increasing the rate at which ATP is regenerated (i.e. when the creatine phosphate kinase content is increased from 0.8 unit to 4 units). In addition, (Fig. 8) the lag is shortened by an excess of ATP. (In the reconstituted system the lag in O_2 evolution may be similarly shortened by the addition of increasing quantities of creatine phosphate kinase in the presence of constant creatine phosphate.) Oxidation of NADPH is also inhibited by Rib-5-*P* but not in the presence of 20 mM DL-glyceraldehyde or in the absence of Rib-5-*P* kinase (cf. Figs. 6 and 7)

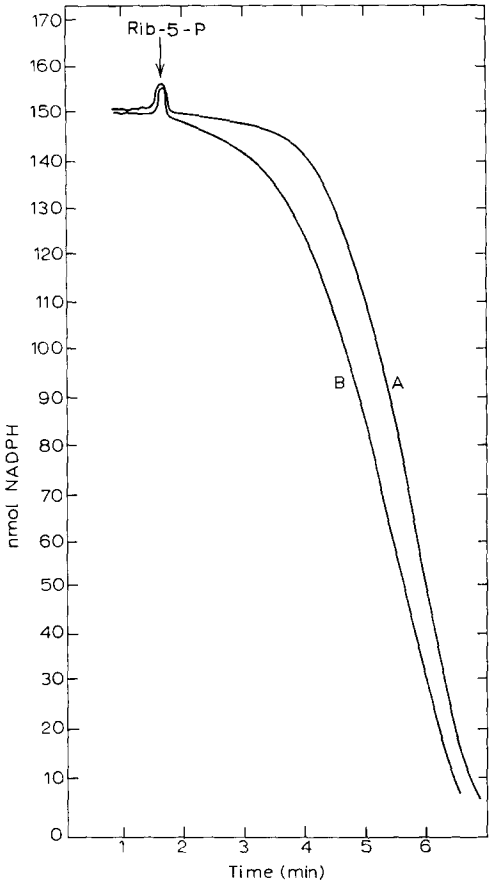


Fig. 8. Effect of two different ATP concentrations on the lag in Rib-5-*P*-dependent NADPH oxidation by chloroplast extract. Reaction mixtures as in Experimental except that in addition they contained 1 mM Rib-5-*P*, 0.8 unit creatine phosphokinase, and A, 0.1 mM ATP; and B, 4 mM ATP.

again strongly implying that Rib-5-*P* acts as an ATP sink after its conversion to Rbu-5-*P*. Similarly Fig. 9 shows that there is no inhibition of the complete system after equilibrium has been attained, i.e. if added Rib-5-*P* has been converted to *sn*-phospho-3-glycerol and ATP regenerated from ADP before the addition of NADPH the final stage of the reaction follows immediately. Collectively all of these figures show that the transient inhibition by Rib-5-*P* of *sn*-phospho-3-glycerol reduction (and its associated O_2 evolution in the reconstituted system) can be attributed to ADP generation in situ.

DISCUSSION

The results confirm the previously reported inhibition of *sn*-phospho-3-glycerol-dependent O_2 evolution by ADP and show that similar responses may be produced by a variety of ATP sinks (which are, in effect, ADP-generating systems). The sequence of events (Fig. 10) may be summarised as follows. Upon illumination

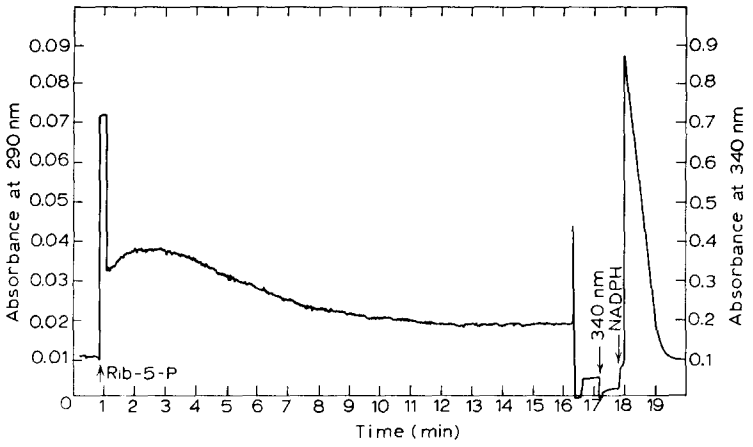


Fig. 9. Spectroscopic trace to demonstrate absence of detectable lag in Rib-5-*P*-dependent NADPH oxidation after pre-equilibration. Reaction mixture as in Experimental but with 4 units creatine phsophokinase, 0.1 mM ATP, and no NADPH. Rib-5-*P* (1 mM) was added as specified and the formation of pentoketose was followed at 290 nm [18]. After equilibration the wavelength was altered to 340 nm and NADPH (0.15 mM) was added as indicated.

electrons are transported from water to NADP with the concomitant evolution of O_2 and formation of ATP. In the reductive pentose phosphate cycle NADPH is oxidised by *sn*-glycerol 1,3-bisphosphate and if this metabolite has to be formed from *sn*-phospho-3-glycerol then ATP must be consumed. Accordingly, O_2 evolution will occur in the presence of a steady-state ATP/ADP ratio as ATP consumption and regeneration come into balance. If the ATP/ADP ratio is decreased, either by the direct addition of ADP or by the introduction of an ADP-generating system, *sn*-phospho-3-glycerol-

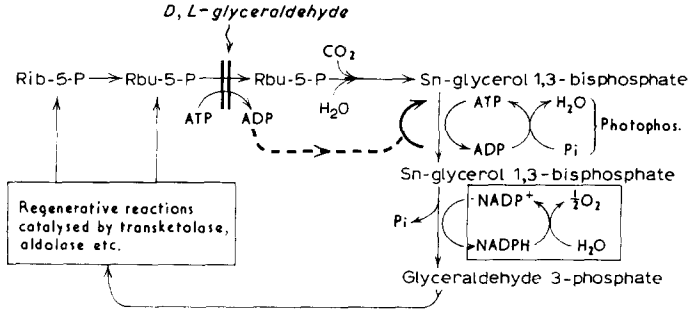


Fig. 10. Simplified representation of reductive pentose phosphate pathway illustrating the nature of Rib-5-*P* inhibition. The box at the right indicates the source of the O_2 which is evolved in Figs. 1–6. In the light, electrons are transported from water to NADP, O_2 is evolved and the ATP required for *sn*-phospho-3-glycerol reduction is generated. In the presence of Rib-5-*P*, ATP is also consumed in the phosphorylation of Rbu-5-*P* and this reaction acts as an ADP generator, displacing the equilibrium (heavy arrow) of the reaction catalysed by *sn*-phospho-3-glycerol kinase. DL-Glyceraldehyde inhibits the Rbu-5-*P* kinase reaction (Slabas and Walker, ref. 12) thus diminishing the Rib-5-*P* inhibition. The Rib-5-*P* inhibition may be simulated by direct addition of ADP or an alternative ADP generator (such as glucose plus hexokinase). Conversely, it is made ineffectual by a powerful ATP generator (such as creatine phosphate plus creatine phosphate kinase).

dependent O_2 evolution will cease as *sn*-glycerol 1,3-bisphosphate is preferentially reconverted to *sn*-phospho-3-glycerol instead of undergoing reduction to glyceraldehyde 3-phosphate. Conversely, these interruptions or delays will be abolished by ATP-generating systems such as creatine phosphate plus its kinase. In the context of photosynthetic carbon assimilation, the most interesting ADP-generating system used in these experiments is that which is brought into operation by the addition of Rib-5-*P*. The immediate and total inhibition of *sn*-phospho-3-glycerol-dependent O_2 evolution caused by the admixture of relatively small quantities of Rib-5-*P* provides a striking example of the way in which one reaction of the cycle can materially effect the course of another, which is (metabolically) some distance removed. Phosphoribulokinase is known [13] to have a much higher affinity for ATP ($K_m = 0.42$ mM) than *sn*-phospho-3-glycerol kinase ($K_m = 2.0$ mM), and this will increase the effectiveness with which the phosphorylation of Rbu-5-*P* will function as an ATP sink. The present results therefore endorse a previous conclusion [14] that the inhibition of *sn*-phospho-3-glycerol-dependent O_2 evolution by Rib-5-*P* in intact, P_i-deficient, chloroplasts could be attributed to consumption of ATP in the reaction catalysed by Rbu-5-*P* kinase although the ADP concentration (and therefore the ATP/ADP ratio) would appear to be more important than the ATP concentration as such. In normal photosynthesis there is a 1 to 1 stoichiometry between O_2 evolved and CO_2 fixed. The transient inhibition brought about by an alteration of the Rib-5-*P* pool shows one way in which this relationship is maintained. The chloroplast envelope permits rapid export of *sn*-phospho-3-glycerol (for reviews see e.g. Heber [15] and Walker [16]) and in many circumstances the cytoplasm and the reductive pentose phosphate cycle will be indirect competition for *sn*-phospho-3-glycerol formed in the carboxylation reaction. If Rib-5-*P* regeneration tended to predominate then the consequent drain on ATP would limit *sn*-phospho-3-glycerol reduction and therefore favour the escape of *sn*-phospho-3-glycerol. If the escape of *sn*-phospho-3-glycerol tended to become excessive the ATP/ADP ratio would increase, a greater proportion of the *sn*-phospho-3-glycerol formed would be reduced and therefore become available for recycling as well as for export.

The results also show that the concept of "photosynthetic control" [17] can be extended beyond electron transport. If the steady-state concentration of ADP is too low, the rate at which NADP is reduced will diminish [17]. If the ADP is too high the rate of reoxidation of NADPH will decrease. In this way the ATP/ADP ratio would appear to exert an exceptionally important regulatory influence, in photosyntheses balancing as it does the requirements of electron transport, carboxylation, reduction and export.

ACKNOWLEDGEMENTS

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REFERENCES

1 Stokes, D. M. and Walker, D. A. (1971) Plant Physiol. 48, 163–165
2 Walker, D. A., McCormick, A. V. and Stokes, D. M. (1971) Nature 233, No. 5318, 346–347

3 Lilley, R. McC., Holborow, K. and Walker, D. A. (1974) New Phytol. 73, 657–662
4 Lilley, R. McC. and Walker, D. A. (1974) Biochim. Biophys. Acta 368, 260–278
5 Slabas, A. R. and Walker, D. A. (1976) Biochem. J. 154, 185–192
6 Rao, K. K., Cammack, R., Hall, D. O. and Johnson, C. E. (1971) Biochem. J. 122, 257–265
7 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
8 Delieu, T. and Walker, D. A. (1972) New Phytol. 71, 201–225
9 Walker, D. A. (1973) New Phytol. 72, 209–235
10 Walker, D. A. and Lilley, R. McC. (1974) Plant Physiol. 54, 950–952
11 Baier, D. and Latzko, E. (1975) Biochim. Biophys. Acta 396, 141–148
12 Slabas, A. R. and Walker, D. A. (1976) Biochem. J. 153, 613–619
13 Lavergne, D., Bismuth, E. and Champigny, M. L. (1974) Plant Sci. Lett. 3, 391–397
14 Cockburn, W., Baldry, C. W. and Walker, D. A. (1967) Biochim. Biophys. Acta 143, 614–624
15 Heber, U. (1974) Annu. Rev. Plant Physiol. 25, 393–421
16 Walker, D. A. (1974) Chloroplast and Cell (Northcote, D. H. ed.), Med. Tech. Publ. Int. Rev. Sci. Bioch., Ser. 1, Vol. 11, pp. 1–49, Butterworths, London
17 West, K. R. and Wiskich, J. T. (1968) Biochem. J. 109, 527–532
18 Wood, T. (1970) Anal. Biochem. 33, 297–306