

## Localization of Inhibition by Adenosine Diphosphate of Phosphoglycerate-Dependent Oxygen Evolution in a Reconstituted Chloroplast System

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ADP was shown to inhibit phosphoglycerate-dependent  $O_2$  evolution in a simplified reconstituted chloroplast system containing 3-phosphoglycerate kinase and triose phosphate dehydrogenase. Rates of  $O_2$  evolution in the simplified system are comparable with those obtained by using stromal protein rather than purified enzymes. ADP does not inhibit  $O_2$  evolution with glycerate 1,3-bisphosphate as substrate nor does it inhibit triose phosphate dehydrogenase. The inhibitory effect of ADP is attributed to an increase in the rate of conversion of glycerate bisphosphate into phosphoglycerate. The results are discussed in terms of control by ADP of phosphoglycerate-dependent oxygen evolution.

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 (see, e.g., Bassham & Calvin, 1957) cycle in the chloroplast stromal compartment. Isolated intact chloroplasts (type A; Hall, 1972) are capable of high rates of  $O_2$  evolution with 3-phosphoglycerate as substrate (Walker & Hill, 1967). Conversely, washed envelope-free chloroplasts (type D; Hall, 1972) will evolve  $O_2$  with 3-phosphoglycerate only if supplied with a chloroplast extract (i.e. water-soluble proteins released by osmotic rupture) and sufficient ferredoxin, NADP<sup>+</sup> and ATP to give a reconstituted system (Stokes & Walker, 1971).  $O_2$  evolution by the reconstituted system may be envisaged as a Hill reaction in which NADP<sup>+</sup> is reduced by electrons from  $H_2O$  and reoxidized by glycerate 1,3-bisphosphate (Lilley & Walker, 1974) (Scheme 1).

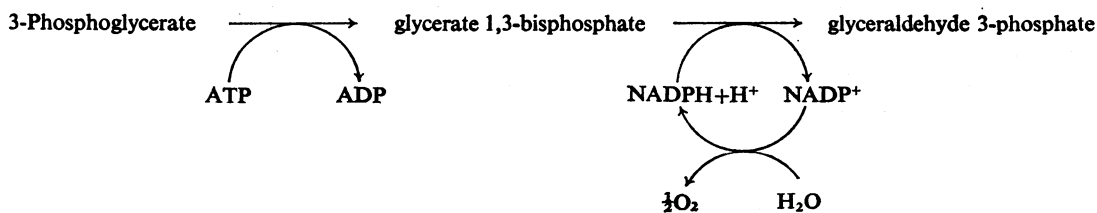
Phosphoglycerate-dependent  $O_2$  evolution in the reconstituted system is subject to inhibition by ADP (Lilley & Walker, 1974). This inhibition could be attributed either to a mass-action effect on the

endergonic reaction catalysed by phosphoglycerate kinase (ATP-3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) or to inhibition of NADP-specific glyceraldehyde 3-phosphate dehydrogenase activity [D-glyceraldehyde 3-phosphate-NADP<sup>+</sup> oxidoreductase (phosphorylating), EC 1.2.1.13] (Lilley & Walker, 1974). In an attempt to define the nature of ADP inhibition in the reconstituted system we have investigated the effect of ADP: (a) on the purified triose phosphate dehydrogenase by a direct spectroscopic assay utilizing glycerate 1,3-bisphosphate as substrate; (b) on a reconstituted chloroplast system containing NADP-specific triose phosphate dehydrogenase and phosphoglycerate kinase, which is capable of phosphoglycerate-dependent  $O_2$  evolution; and (c) on an otherwise similar reconstituted system containing only NADP<sup>+</sup>-specific triose phosphate dehydrogenase and utilizing glycerate 1,3-bisphosphate as substrate.

### Experimental

#### Materials

Spinach (*Spinacia oleracea* var. True Hybrid 102; Arthur Yates and Co., Sydney, N.S.W., Australia)



Scheme 1.

was grown as described by Lilley & Walker (1974). NADPH, NAD<sup>+</sup>, GSH, Tris and yeast alcohol dehydrogenase were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. NADP<sup>+</sup>, ATP, ADP, AMP, glyceraldehyde 3-phosphate, 3-phosphoglyceric acid, rabbit-muscle triose phosphate dehydrogenase and yeast phosphoglycerate kinase were purchased from Boehringer Corp. (London) Ltd., Ealing, London W5 2TZ, U.K. Sephadex was obtained from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. DEAE-cellulose was obtained from Whatman Ltd., Maidstone, Kent, U.K. Ferredoxin was prepared as described by Rao *et al.* (1971). All remaining reagents were purchased from Fisons Ltd., Loughborough, Leics., U.K., and were of the highest purity available.

*Intact chloroplasts, chloroplast extract and thylakoids.* These were prepared by the procedure described by Lilley & Walker (1974) except that the envelope-free chloroplasts were washed by resuspending them in 50 ml of full-strength resuspension medium and centrifuged for 10 min at 13000g before resuspension.

*Glycerate 1,3-bisphosphate.* This was prepared by the method of Negelein (1957, 1965), except that additions of acetaldehyde (0.1  $\mu$ mol) were made after 2 and 4 min of incubation. The neutralized filtrate was used for experimental purposes.

#### Assays

*Protein.* This was measured by the Lowry method, as modified by Bailey (1962), after precipitation in 5% (w/v) trichloroacetic acid and resuspension in 5% (w/v) NaOH. Bovine serum albumin, fraction 5 (Sigma), previously dried in a desiccator, was used as a standard.

*Chlorophyll.* This was measured as described by Arnon (1949).

#### Enzyme assays

Spectroscopic assays were performed by following the rate of change of  $E_{340}$  at 20°C. An enzyme unit is defined as that quantity which will convert 1  $\mu$ mol of substrate into product/min under the conditions specified.

*NADP<sup>+</sup>-specific triose phosphate dehydrogenase.* Unless otherwise stated this was assayed indirectly in a mixture containing the following reactants in a final volume of 1 ml: 100 mM-Tris/HCl buffer, pH 8.0, 10 mM-MgCl<sub>2</sub>, 10 mM-GSH, 6 mM-ATP, 0.12 mM-NADPH, 6 mM-phosphoglycerate and 4 units of phosphoglycerate kinase. The reaction was initiated by the addition of 10  $\mu$ l of appropriately diluted enzyme.

Alternatively, NADP<sup>+</sup>-specific triose phosphate dehydrogenase was assayed directly by using glycer-

ate 1,3-bisphosphate as substrate as described in the legends to the Figures.

*Phosphoglycerate kinase.* This was assayed in a reaction mixture, final volume 1 ml, containing 100 mM-Tris/HCl buffer, pH 8.0, 10 mM-MgCl<sub>2</sub>, 10 mM-GSH, 6 mM-ATP, 0.14 mM-NADH, 6 mM-phosphoglycerate and 2 units of NAD<sup>+</sup>-specific triose phosphate dehydrogenase. The reaction was initiated by the addition of 10  $\mu$ l of appropriately diluted enzyme.

*O<sub>2</sub> evolution by the reconstituted chloroplast system.* This was measured polarographically by using twin Hansatech electrodes (Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk, U.K.) as described by Delieu & Walker (1972). The reaction mixtures were the same as those used by Lilley & Walker (1974) except that for the simplified system 3 units of NADP<sup>+</sup>-specific triose phosphate dehydrogenase and 55 units of phosphoglycerate kinase were substituted for the chloroplast extract. With glycerate 1,3-bisphosphate as substrate, O<sub>2</sub> evolution was measured in the presence of 3 units of NADP<sup>+</sup>-specific triose phosphate dehydrogenase and 1.4  $\mu$ mol of glycerate 1,3-bisphosphate, but in the absence of phosphoglycerate and chloroplast extract. All O<sub>2</sub> measurements were made at 20°C in a final volume of 1 ml.

#### Purification of NADP<sup>+</sup>-specific triose phosphate dehydrogenase

All operations were carried out between 0 and 4°C unless otherwise specified. Centrifugation was performed at 13000g. Spinach (2 kg) was homogenized for 1 min (Polytron blender) in 2 litres of 0.2 M-potassium phosphate buffer, pH 9.0, containing 30 mM-EDTA (disodium salt). After centrifugation for 30 min, 300 ml quantities of the supernatant were rapidly heated to 60°C (boiling-water bath) and were maintained at this temperature for 25 min. The solution was rapidly cooled and then centrifuged for 30 min. The supernatant was adjusted to 45% saturation by the addition of solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and after the pH had been re-adjusted to pH 8.5 with KOH the solution was centrifuged for 20 min. The precipitate was discarded and solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> added to adjust the supernatant to 60% saturation. The solution was left for 20 min and was then centrifuged for 30 min. The precipitate was resuspended in 40 ml of 0.3 M-potassium phosphate buffer, pH 9.0, containing 90 mM-EDTA and 1 mM- $\beta$ -mercaptoethanol. At this stage the protein could be stored at -18°C with little loss of activity. The suspension was made 1 mM with respect to cysteine hydrochloride, and after the addition of 0.8 vol. of acetone at -20°C was centrifuged at -18°C for 10 min. The precipitate was resuspended in 40 ml of 25 mM-potassium phosphate buffer, pH 8.0,

containing 2.5 mM-EDTA and 1 mM- $\beta$ -mercaptoethanol. The solution was stirred for 10 min and then centrifuged for 10 min. The straw-yellow supernatant was retained and applied to a DEAE-cellulose column (4 cm  $\times$  30 cm) previously equilibrated with 25 mM-potassium phosphate buffer, pH 8.0, containing 2.5 mM-EDTA and 1 mM- $\beta$ -mercaptoethanol and eluted with the same buffer. Fractions containing triose phosphate dehydrogenase activity were pooled and the protein was precipitated by the addition of  $(\text{NH}_4)_2\text{SO}_4$  (0.42 g/ml). The precipitate was collected by centrifugation for 20 min and resuspended in the same buffer used for homogenization.

The sample was applied to a Sephadex G-200 column (2.5 cm  $\times$  110 cm) previously equilibrated with 100 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-EDTA and 1 mM- $\beta$ -mercaptoethanol and eluted with the same buffer. The bulk of the triose phosphate dehydrogenase activity emerged before the phosphoglycerate kinase, and fractions free of kinase activity were pooled and the protein was

precipitated by the addition of  $(\text{NH}_4)_2\text{SO}_4$  (0.42 g/ml). The precipitate was collected by centrifugation for 30 min and resuspended in homogenization buffer containing 2 mg of bovine serum albumin/ml. The preparation contained less than 0.1% phosphoglycerate kinase activity and was dialysed as a routine (2 ml of enzyme against 4 litres of 50 mM-Tris/HCl buffer, pH 8, containing 5 mM-EDTA and 1 mM-dithiothreitol for 12 h) before use. The enzyme showed no detectable loss in activity when stored for 1 month as the  $(\text{NH}_4)_2\text{SO}_4$  precipitate in a deep freeze. Table 1 summarizes the purification procedure.

## Results

### Linearity of spectroscopic assays

The spectroscopic assay of  $\text{NADP}^+$ -dependent triose phosphate dehydrogenase gave a linear relationship between rate and enzyme concen-

Table 1. Purification of  $\text{NADP}^+$ -dependent triose phosphate dehydrogenase

Stage	Total volume (ml)	Triose phosphate dehydrogenase (units)	Phosphoglycerate kinase (units)	Triose phosphate dehydrogenase specific activity (enzyme units/mg of protein)	Ratio of triose phosphate dehydrogenase/3-phosphoglycerate kinase
Crude homogenate	2875	7800	79 500	0.5	0.1
Thermal supernatant	2620	8000	514	1.2	15.7
45% Satd. $(\text{NH}_4)_2\text{SO}_4$ supernatant	2940	8250	676	7.7	12.2
60% Satd. $(\text{NH}_4)_2\text{SO}_4$ ppt.	44	8800	91	—	96.4
Acetone fraction	40	5800	69	21.6	84.0
DEAE-cellulose eluate	110	7200	442	31.2	16.3
Sephadex G-200 eluate	52	3000	1.7	46.2	1750

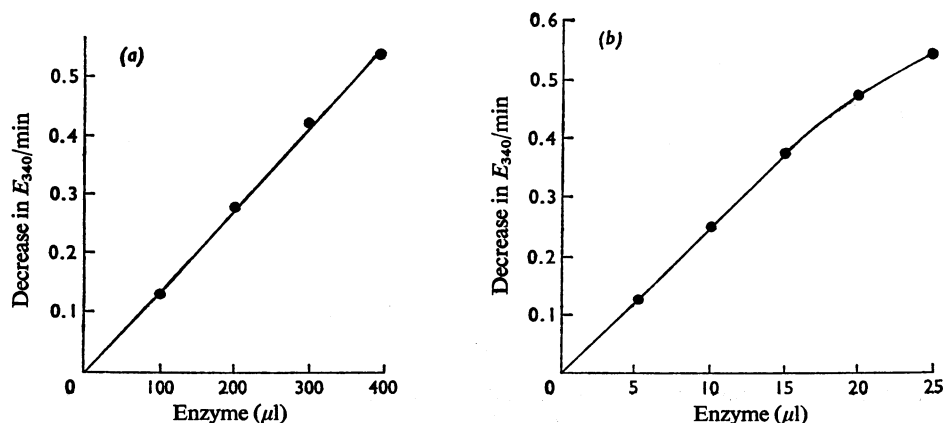


Fig. 1. Linearity of assays for (a)  $\text{NADP}^+$ -dependent triose phosphate dehydrogenase and (b) phosphoglycerate kinase

Assay conditions are given in the Experimental section.

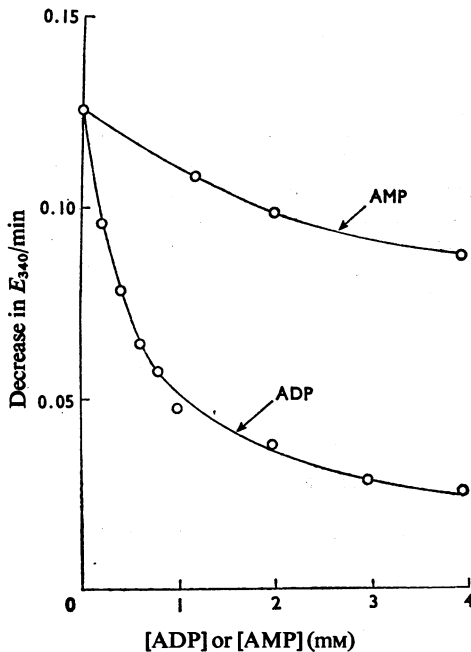


Fig. 2. Inhibition of triose phosphate dehydrogenase in the linked enzyme assay

The reaction velocity is plotted as a function of [ADP] and [AMP]. The assay conditions were as described in the Experimental section, except that the [ATP] was 4 mM.

tration up to a decrease in  $E_{340}$  of 0.55/min (Fig. 1a). The spectroscopic assay of phosphoglycerate kinase was similarly linear with enzyme concentration up to a decrease in  $E_{340}$  of 0.38/min (Fig. 1b).

*Effect of ADP and AMP on triose phosphate dehydrogenase assayed by the indirect spectroscopic method*

Both ADP and AMP inhibited NADPH oxidation in the linked assay (Fig. 2). As shown previously with chloroplast extract (Lilley & Walker, 1974) ADP was a much stronger inhibitor than AMP. This effect cannot be ascribed to chelation of free  $\text{Mg}^{2+}$  by the ADP or AMP, since in these experiments a much higher concentration of  $\text{Mg}^{2+}$  was used, resulting in a large excess of free  $\text{Mg}^{2+}$ . In agreement with Lilley & Walker (1974), increased phosphoglycerate kinase did not alleviate this inhibition but slightly increased it (Fig. 3). The inhibition was, however, alleviated by increased  $\text{NADP}^+$ -specific triose phosphate dehydrogenase. Such alleviation could result from a decrease in the steady-state concentration of glycerate 1,3-bisphosphate as a result of its accelerated reduction to glyceraldehyde 3-phosphate.

*Effect of ADP on  $\text{NADP}^+$ -specific triose phosphate dehydrogenase activity assayed by the direct method*

$\text{NADP}^+$ -linked triose phosphate dehydrogenase activity was not inhibited by ADP (4 mM) when

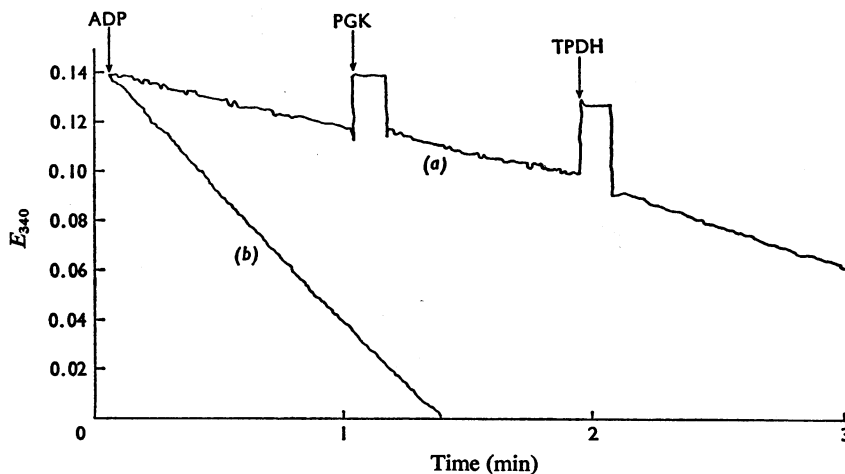


Fig. 3. Effect of ADP on the (indirect) spectroscopic linked assay for  $\text{NADP}^+$ -specific triose phosphate dehydrogenase (a) with 4 mM-ADP and (b) without ADP

Phosphoglycerate kinase (40 units) and triose phosphate dehydrogenase (0.03 unit) were added as specified. Assay conditions were as described in the Experimental section except that the [ATP] was 4 mM. Abbreviations: PGK, 3-phosphoglycerate kinase; TPDH, triose phosphate dehydrogenase.

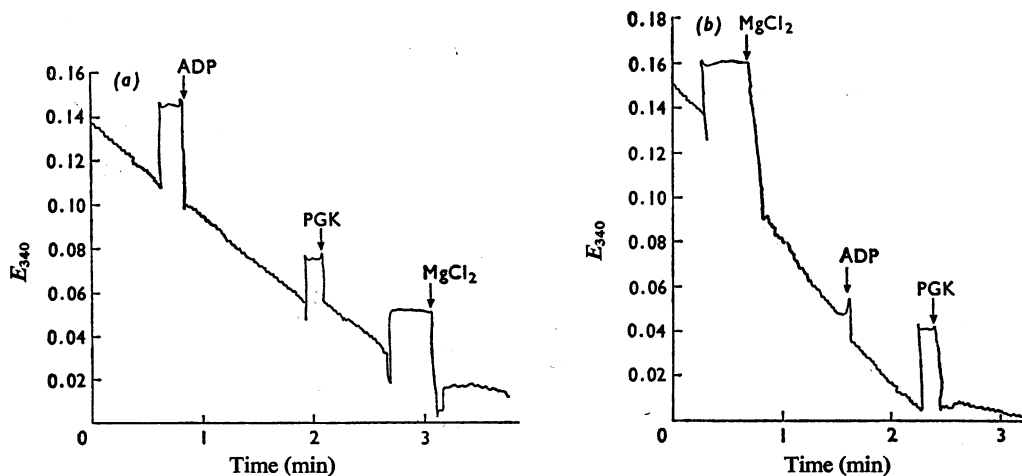


Fig. 4. Effect of ADP and  $Mg^{2+}$  on the direct spectroscopic assay of  $NADP^+$ -linked triose phosphate dehydrogenase

The assay mixture, final volume 1 ml, contained 4 mM-ATP, 100 mM-Tris/HCl buffer, pH 8.0, 10 mM-GSH, 6 mM phosphoglycerate, 0.14 mM-NADPH, 0.06  $\mu$ mol of glycerate 1,3-bisphosphate and 15  $\mu$ mol of EDTA. The reactions were initiated by the addition of triose phosphate dehydrogenase: 4  $\mu$ mol of ADP, 40 units of phosphoglycerate kinase and 75  $\mu$ mol of  $MgCl_2$  were added as indicated. Note that (a) and (b) differ in the order of additions. The presence of 3-phosphoglycerate kinase produces no inhibition unless its cofactor  $Mg^{2+}$  is also present. In (b)  $Mg^{2+}$  and ADP were present before the addition of 3-phosphoglycerate kinase. In (a) inhibition follows the addition of  $Mg^{2+}$ . Abbreviation: PGK, 3-phosphoglycerate kinase.

assayed directly. Addition of phosphoglycerate kinase was also without effect unless  $Mg^{2+}$  was present (Fig. 4a). Fig. 4(b) shows that Mg-ADP did not inhibit triose phosphate dehydrogenase activity. Subsequent addition of phosphoglycerate kinase brought about a marked inhibition (presumably because of increased phosphoglycerate formation).

#### Effect of ADP on phosphoglycerate-dependent $O_2$ evolution in the reconstituted chloroplast system

As shown by Lilley & Walker (1974), phosphoglycerate-dependent  $O_2$  evolution in the reconstituted chloroplast system was inhibited by ADP (Fig. 5a). A large inhibition was obtained by the addition of 2  $\mu$ mol of ADP. If  $NADP^+$ -dependent triose phosphate dehydrogenase and phosphoglycerate kinase were substituted for chloroplast extract (Figs. 5b and 5c) the rate of phosphoglycerate-dependent  $O_2$  evolution was almost the same as that observed in the reconstituted system (Fig. 5a). Both ADP and nigericin inhibit phosphoglycerate-dependent  $O_2$  evolution in this simplified reconstituted system and this inhibition can be reversed by the addition of creatine phosphate and creatine kinase. Since ADP affects the simplified reconstituted system and not triose phosphate dehydrogenase, it seemed clear that it must inhibit either electron transport

(which would be entirely contrary to general experience) or the reaction catalysed by phosphoglycerate kinase.

#### Effect of ADP on $O_2$ evolution with glycerate 1,3-bisphosphate as substrate

Fig. 6 shows that 4 mM-ADP was without effect on  $O_2$  evolution with glycerate 1,3-bisphosphate as substrate, thus eliminating the possibility that ADP might inhibit at some site not involved in the formation of this compound from 3-phosphoglycerate.

#### Discussion

Lilley & Walker (1974) viewed phosphoglycerate-dependent  $O_2$  evolution by a reconstituted chloroplast system as a Hill reaction in which  $NADP^+$  was reduced by electrons from water and reoxidized by glycerate 1,3-bisphosphate. The simplified reconstituted system reported here (in which chloroplast extract is replaced by purified enzymes) is in accord with this view and is capable of comparable rates of  $O_2$  evolution and exhibits similar sensitivity to AMP and ADP. Pupillo *et al.* (1971) have reported that spinach chloroplast  $NADP^+$ -specific triose phosphate dehydrogenase, assayed in the direction

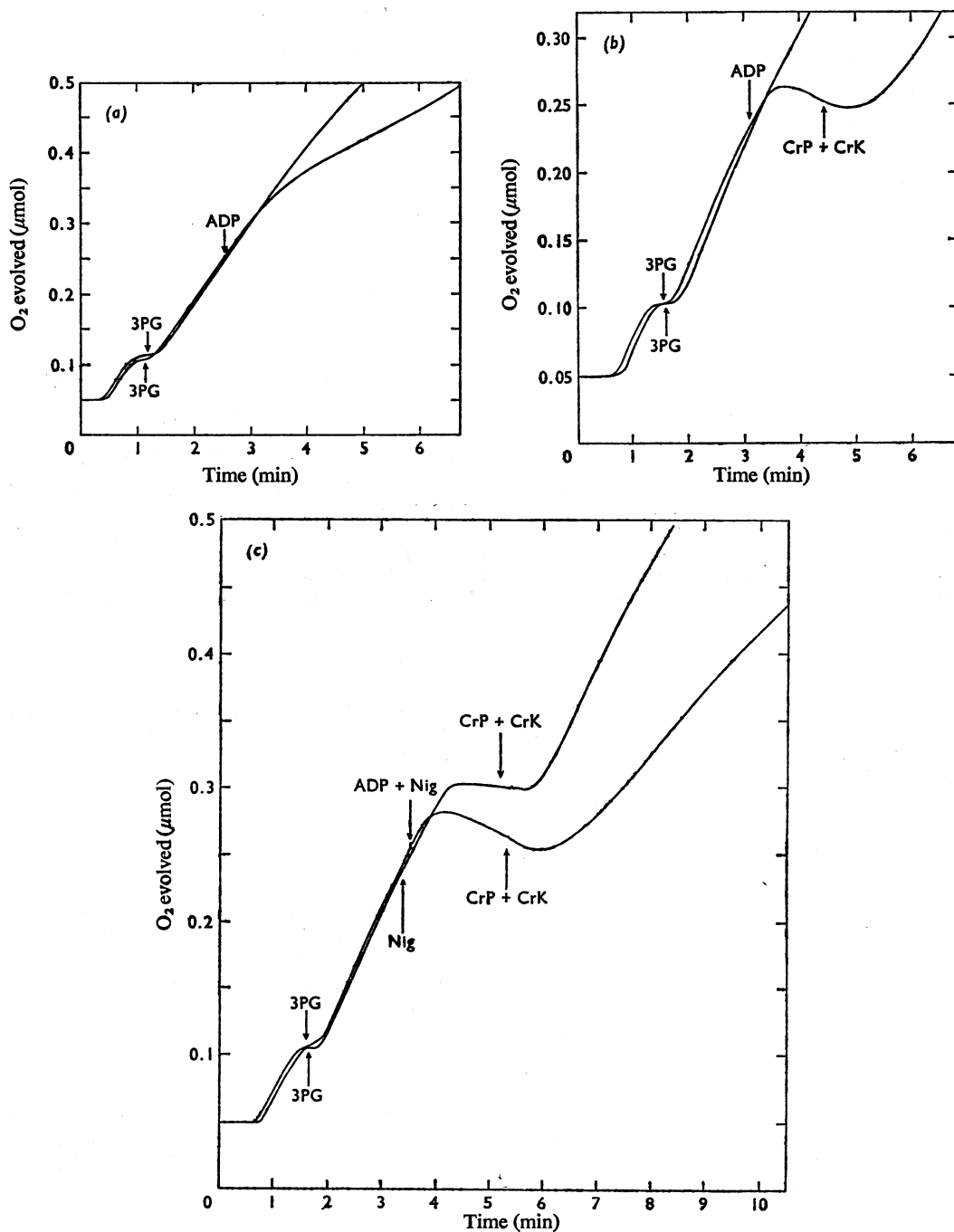


Fig. 5. Inhibition of photosynthetic  $O_2$  evolution by ADP and an uncoupler

(a) Effect of 2 mM-ADP on phosphoglycerate-dependent  $O_2$  evolution in the reconstituted chloroplast system. The reaction mixture was as described in the Experimental section but initially without the phosphoglycerate. 1  $\mu\text{mol}$  of phosphoglycerate and 2  $\mu\text{mol}$  of ADP were added as indicated. (b) Effect of 4 mM-ADP on phosphoglycerate-dependent  $O_2$  evolution in the simplified reconstituted system. The reaction mixture was as described in the Experimental section but initially without the phosphoglycerate: 1  $\mu\text{mol}$  of phosphoglycerate and 4  $\mu\text{mol}$  of ADP were then added as indicated. The effect of ADP was

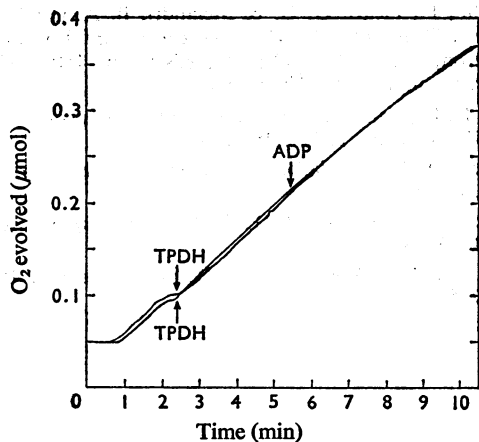


Fig. 6. Effect of ADP ( $4\mu\text{mol}$ ) on  $\text{O}_2$  evolution with glycerate 1,3-bisphosphate as substrate

The reaction conditions were as described in the Experimental section, except that triose phosphate dehydrogenase (TPDH; 2.9 units) and ADP ( $2\mu\text{mol}$ ) were not added until indicated. It should be noted that this reaction system contained no phosphoglycerate kinase.

of  $\text{NADP}^+$  reduction, is inhibited by adenine nucleotides. Utilizing both a direct assay for the  $\text{NADP}^+$ -specific triose phosphate dehydrogenase and  $\text{O}_2$  evolution in a simplified reconstituted system with glycerate 1,3-bisphosphate as substrate, we have not found any effect of ADP on triose phosphate dehydrogenase assayed in the direction of  $\text{NADPH}$  oxidation.

Phosphoglycerate-dependent  $\text{O}_2$  evolution is inhibited by ADP both in the reconstituted chloroplast system (Lilley & Walker, 1974) and in the simplified reconstituted system used here. Lilley & Walker (1974) suggested that ADP must inhibit one or other of the steps in the reduction of phosphoglycerate to triose phosphate. Since  $\text{O}_2$  evolution with glycerate 1,3-bisphosphate as substrate is not inhibited (Fig. 6), there seems no doubt that the site of action must be the reaction catalysed by phosphoglycerate kinase. Pacold & Anderson (1973) have reported that pea chloroplast phosphoglycerate kinase is inhibited by ADP when assayed

in the ATP-utilizing direction and is stimulated by ADP when assayed in the ATP-synthesizing direction. Lavergne *et al.* (1974) have also reported that spinach chloroplast phosphoglycerate kinase is inhibited by ADP when assayed in the ATP-utilizing direction. From the present results we conclude that ADP inhibits phosphoglycerate-dependent  $\text{O}_2$  evolution in the reconstituted chloroplast system by increasing the rate of phosphoglycerate formation from glycerate 1,3-bisphosphate. Since the concentration of  $\text{Mg}^{2+}$  used in the experiments reported in the present paper was high ( $10\text{mM}$ ) in comparison with that of adenylate, this effect cannot be attributed to chelation.

Photosynthetic electron transport is accelerated by ADP and inhibited by ATP (West & Wiskich, 1968). Conversely, the present results re-emphasize the readiness with which ATP consumption (and hence ADP accumulation) can slow the reduction of 3-phosphoglycerate to glyceraldehyde 3-phosphate in the Benson-Calvin cycle. Together these two processes must constitute a delicately poised regulatory mechanism which will help to ensure that the rate of ATP production in photosynthesis will respond to the rate at which it is consumed in the Benson-Calvin cycle and vice versa.

#### Note Added in Proof (Revised 25 November 1975)

Bamberger & Avron (1975) have independently concluded that DL-glyceraldehyde acts at (a) a more sensitive site in the conversion of fructose bisphosphate into ribulose 5-phosphate and (b) a less sensitive site in the conversion of ribulose 5-phosphate into ribulose bisphosphate.

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reversed, as indicated, by the addition of  $5\mu\text{mol}$  of creatine phosphate and 4 units of creatine kinase. (c) Effect of nigericin and ADP on phosphoglycerate-dependent  $\text{O}_2$  evolution in the simplified reconstituted system. The reaction mixture was as described in the Experimental section, but initially without phosphoglycerate: phosphoglycerate ( $1\mu\text{mol}$ ), nigericin ( $1\mu\text{mol}$ ) and ADP ( $4\mu\text{mol}$ ) were added as indicated. The ADP inhibition was reversed by the addition of creatine phosphate + creatine kinase, as in Fig. 5(b). Abbreviations: 3PG, 3-phosphoglycerate; CrP, creatine phosphate; CrK, creatine kinase; Nig, nigericin.

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