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PHOTOSYNTHESIS IN A RECONSTITUTED CHLOROPLAST SYSTEM FROM SPINACH

SOME FACTORS AFFECTING CO_2-DEPENDENT OXYGEN EVOLUTION WITH FRUCTOSE-1,6-BISPHOSPHATE AS SUBSTRATE

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SUMMARY

When envelope-free spinach chloroplasts are incubated with stromal protein, catalytic NADP, catalytic ADP, radioactive bicarbonate and fructose 1,6-bisphosphate, $^{14}CO_2$ fixation starts immediately upon illumination but oxygen evolution is delayed. The delay is increased by the addition of fructose 6-phosphate and by a variety of factors known (or believed) to increase fructose bisphosphatase activity (such as dithiothreitol, more alkaline pH, higher [Mg] and antimycin A). Conversely, the lag can be decreased or eliminated by the addition of an ATP-generating system. Bearing in mind the known inhibition, by ADP, of *sn*-phospho-3-glycerate (3-phosphoglycerate) reduction it is concluded that the lag in O_2 evolution results from the production of ribulose 5-phosphate from fructose bisphosphate and that this in turn inhibits the reoxidation of NADPH by adversely affecting the ADP/ATP ratio. The results are discussed in their relation to the mode of action of antimycin A and to regulation of the reductive pentose phosphate pathway.

INTRODUCTION

When isolated chloroplasts are osmotically shocked they lose almost all of their ability to assimilate carbon photosynthetically [1–3] and to support O_2 evolution with *sn*-phospho-3-glycerate (3-phosphoglycerate) as substrate [4]. These functions can be restored [5–11] by the addition of ferredoxin, NADP, ADP, Mg^{2+} and "chloroplast extract" (unfractionated stromal protein). With *sn*-phospho-3-glycerate as substrate, O_2 evolution is immediate and there is no appreciable lag in either O_2 evolution or CO_2 fixation when Rbu-1,5-P_2 and CO_2 are substituted for *sn*-phospho-

Abbreviations: Rib-5-*P*, ribose 5-phosphate; Rbu-5-*P*, ribulose 5-phosphate; Rbu-1,5-P_2, ribulose 1,5-bisphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-*P*, fructose 6-phosphate.

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3-glycerate. With Rib-5-*P*, however, there is a lag [7, 9], which increases with increasing Rib-5-*P* concentration [12]. Similarly the onset of *sn*-phospho-3-glycerate-dependent O_2 evolution can be delayed by the presence of Rib-5-*P* or interrupted by the addition of Rib-5-*P* while it is in progress. Because these delays and interruptions can be mimicked by the addition of further ADP (or by ADP-generating systems) and abolished by ATP-generating systems, they have been explained [12] in terms of the known ADP inhibition of *sn*-phospho-3-glycerate reduction. In short, the response to Rib-5-*P* is seen to result from its conversion to Rbu-5-*P* and the consequent action of this metabolite as an ATP sink in the presence of phosphoribulokinase. The present paper reports a similar lag with Fru-1,6-P_2 and attributes it to the same underlying cause (i.e. to an adverse effect on the ATP/ADP ratio following the formation of Rbu-5-*P*). In this instance, however, the results are believed to throw an important light on the way in which fructose-1,6-bisphosphatase activity can influence subsequent reactions in the reductive pentose phosphate pathway and are discussed in this context.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* United States Hybrid 424, Ferry-Morse Seed Co., P. O. Box 100, Mountain View, California) was grown in water culture according to Lilley and Walker [11].

Intact chloroplasts were prepared as before (see e.g. Lilley and Walker [11]), from pre-illuminated spinach, in 0.33 M sorbitol, 5 mM $MgCl_2$, 2 mM sodium isoascorbate and 10 mM $Na_4P_2O_7$ at pH 6.5.

Chloroplast extract. Stromal protein (chloroplast extract) was released from intact chloroplasts by resuspending pellets containing approx. 3 mg chlorophyll in 8 ml of 1/25 dilution medium (below) containing 3 mM dithiothreitol and centrifuging for 10 min at 13 000 $\times g$ [11]. The precipitate was used as envelope-free chloroplasts (below) and the supernatant (chloroplast extract) dialysed (under reduced pressure at 0 °C for 45 min, using a Sartorius membrane) against 1/10 dilution resuspending medium. It was found that the activity of the chloroplast extract fraction could be improved by the inclusion of $MgCl_2$, 5 mM, $NaHCO_3$, 5 mM and dithiothreitol, 1 mM, in the dialysis medium and this procedure was adopted for Figs. 1–8.

Envelope-free chloroplasts obtained in the preparation of chloroplast extract were resuspended in full-strength resuspending medium containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES) at pH 7.9 [11].

Assays. All assays were carried out at 20 °C in full-strength resuspending medium containing the additional additives listed.

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 $W \cdot m^{-2}$.

Chlorophyll was measured according to Arnon [13].

O_2 evolution was measured polarographically as described by Delieu and Walker [14] using twin, Clark-type electrodes purchased from Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk, U.K. Reaction mix-

tures (1 ml except for Fig. 1 which was 2 ml) contained resuspending medium plus the following additions (except where otherwise stated). Envelope-free chloroplasts ≡ 100 μg chlorophyll, chloroplast extract ≡ 200 μg chlorophyll, 0.1 mM NADP, 0.2 mM ADP, 2 mM K_2HPO_4, 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 4 mM sodium isoascorbate, 90 μg ferredoxin, 110 units catalase. Fru-1,6-P_2 (2 mM) was added immediately prior to illumination unless otherwise stated.

CO_2 fixation. This was measured in the same mixture used for O_2 determinations except that volume was increased from 1 to 2 ml and therefore other components of the reaction mixture were increased pro rata. The bicarbonate contained 100 μCi ^{14}C. Samples (10 μl) were removed at intervals and injected into 40 μl of 0.3 M HCl + 10 mM sucrose. Two 20-μl aliquots of this acidified mixture were dried on lens tissue discs (on planchettes) for determination of radioactivity (Nuclear Chicago, gas-flow planchette counter). Standard [^{14}C]sucrose (Amersham) was used as a reference to convert counts recorded to μmol CO_2 fixed.

Ferredoxin was prepared by the method of Rao et al. [15].

Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolyase, EC 3.1.3.11) was prepared by the method of Buchanan et al. [16] and assayed at 20 °C by the method of Latzko et al. [17]. The reaction mixture (final volume 1 ml) contained 100 μmol Tris · HCl buffer (pH 8.8), 10 μmol $MgCl_2$, 2 μmol EDTA, 0.2 μmol NADP, 1.0 unit glucose-6-phosphate dehydrogenase, 1.5 units glucose phosphate isomerase and 0.6 μmol of Fru-1,6-P_2. The reaction was initiated by the addition of 10 μl of appropriately diluted enzyme and followed by the increase in absorbance at 340 nm.

Reagents. $NaH^{14}CO_3$ was purchased from The Radiochemical Centre, Amersham, U.K. Biochemicals and enzymes were purchased from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Antimycin A was purchased from Calbiochem Ltd., London, U.K. and was dissolved in absolute ethanol. All remaining reagents were purchased from Fisons Ltd., Loughborough, and were of the highest purity available.

RESULTS

The reconstituted chloroplast system

As indicated in Methods this is essentially a mixture of envelope-free chloroplasts, stromal protein, coenzymes (NADP and ADP) and cofactors (such as Mg^{2+}). At the concentrations of stromal protein used this system will not fix measurable quantities of CO_2 or evolve measurable quantities of O_2 (other than that associated with the reduction of the catalytic NADP present) except in the presence of a substrate that will lead to the reoxidation of NADPH [5–7, 9–11]. Substrates such as glutathione (in the presence of glutathione reductase) *sn*-glycerate 1,3-bisphosphate, *sn*-phospho-3-glycerate and Rbu-1,5-P_2 (in the presence of CO_2) lead to immediate and continuous O_2 evolution (see, for example, Lilley et al. [9]).

With *sn*-phospho-3-glycerate as substrate O_2 evolution is inhibited by ADP [11] and by reactions which will convert ATP into ADP (including the phosphorylation of Rbu-5-*P* to Rbu-1,5-P_2). Carbon dioxide fixation (itself indicative of *sn*-phospho-3-glycerate formation) starts immediately with Rib-5-*P* as substrate but there is a lag in O_2 evolution [7, 11], which may be abolished by the addition of an

excess of creatine phosphate+creatine phosphokinase (ATP : creatine *N*-phosphotransferase, EC 2.7.3.2) [12]. For this and other reasons the lag in O_2 evolution which is observed with Rib-5-*P* is attributed to ADP inhibition of *sn*-phospho-3-glycerate reduction [12].

The relationship between O_2 evolution and CO_2 fixation with Fru-1,6-P_2 as substrate

Under some conditions the kinetics of CO_2 fixation and CO_2-dependent oxygen evolution with Fru-1,6-P_2 as substrate are similar to those observed with Rib-5-*P* [12]. In general, the relationship illustrated (In Fig. 1) is typical of many experimental situations. It will be seen that whereas CO_2 fixation starts immediately upon illumination the course of O_2 evolution is more complex. The initial O_2 evolution is associated with the reduction of the catalytic NADP present in the mixture. Thereafter there is a lag of several minutes before O_2 evolution reaches its maximum.

Because the kinetics of CO_2 fixation and O_2 evolution with Fru-1,6-P_2 resemble so closely those recorded with Rib-5-*P* it seemed probable that they must also reflect ADP inhibition of *sn*-phospho-3-glycerol reduction and that the lag in O_2

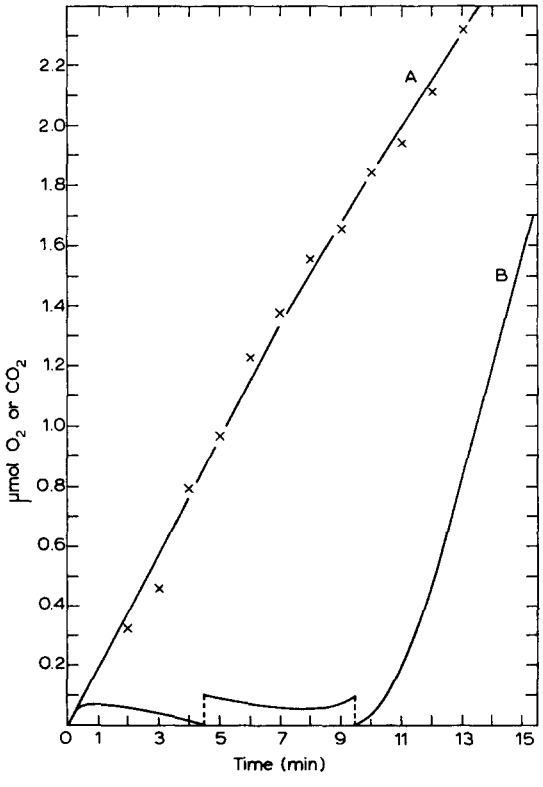

Fig. 1. Simultaneous measurement of photosynthetic $^{14}CO_2$ fixation and O_2 evolution in the reconstituted chloroplast system with Fru-1,6-P_2 as substrate. Reaction mixtures according to Methods. (A) $^{14}CO_2$ fixation, (B) O_2 evolution. (To facilitate recording or representation, traces in this and other figures have been vertically displaced as indicated by the broken vertical lines.)

evolution must therefore be related (a) to the rate at which Fru-1,6-P_2 is converted to Fru-6-*P* and (b) to the effect of this conversion and subsequent events on the steady-state concentration of ADP. On this basis it could be predicted that an increase in the rate of formation of Rib-5-*P* (and hence an increase in ATP consumption) would lead to an increase in the length of the lag and that, conversely, increased ATP formation would diminish it. The extent to which these predictions were borne out is illustrated below in Figs. 1–8.

Factors expected to accelerate Fru-6-P formation

As indicated above, any factor which could facilitate the conversion of Fru-1,6-P_2 to Fru-6-*P* might be expected to extend the lag in O_2 evolution and vice versa. The following were therefore investigated.

1. *Increased substrate.* Fig. 2 shows that the lag in O_2 evolution was approx. 1.2 min with 0.3 mM Fru-1,6-P_2 and that it was increased to approx. 6.5 min when the Fru-1,6-P_2 was increased to 2 mM. This increase in lag with increasing Fru-1,6-P_2 was observed only when the rate of phosphorylation was limiting (i.e. in the presence of relatively low ferredoxin and in the absence of creatine phosphate and creatine phosphokinase; see below). Lag extension was normally accompanied by an increase in the final rate (see, for example, Fig. 3), but during prolonged lags inactivation can occur and this could account for the slightly lower final rate observed (Fig. 2B) in this instance.

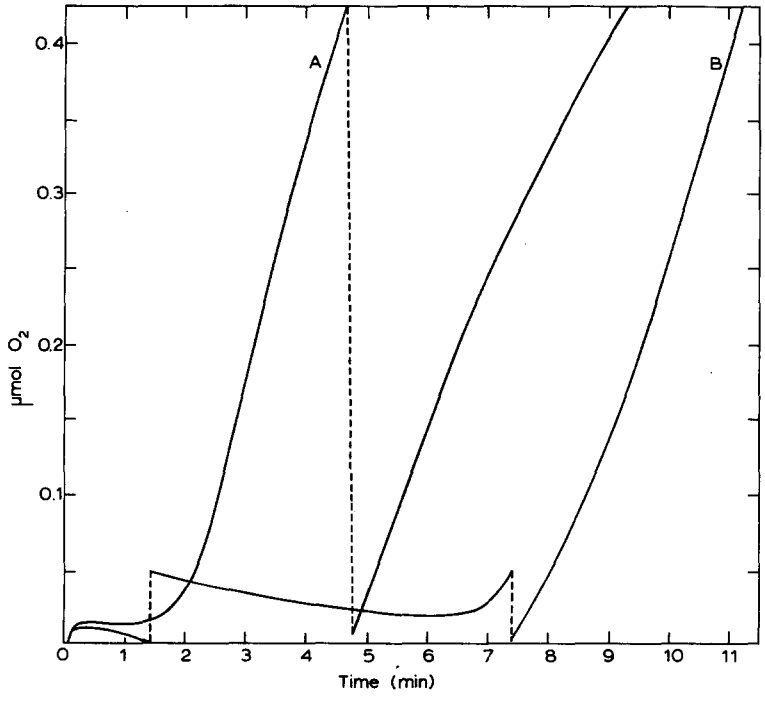

Fig. 2. The effect of increased substrate on the lag in photosynthetic O_2 evolution. (A) 0.3 mM Fru-1,6-P_2, (B) 2 mM Fru-1,6-P_2. Otherwise standard reaction mixtures except for the presence of 5 mM dithiothreitol.

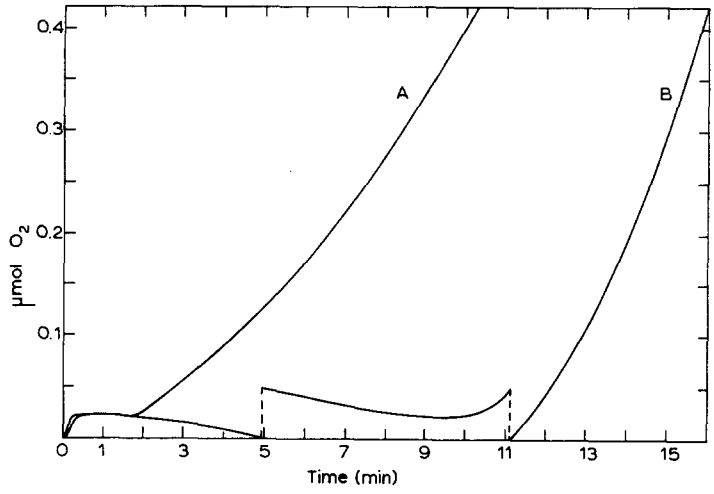

Fig. 3. Lag extension by increased stromal protein. Reaction mixture B contained twice as much chloroplast extract (1.78 mg ≡ 200 µg chlorophyll) as A (0.89 mg ≡ 100 µg chlorophyll). Reaction mixtures otherwise standard except that each contained 5 mM dithiothreitol. The equivalence between chloroplast extract and chlorophyll is an uncorrected value and refers to the protein actually released in this experiment from intact chloroplasts when they were osmotically shocked.

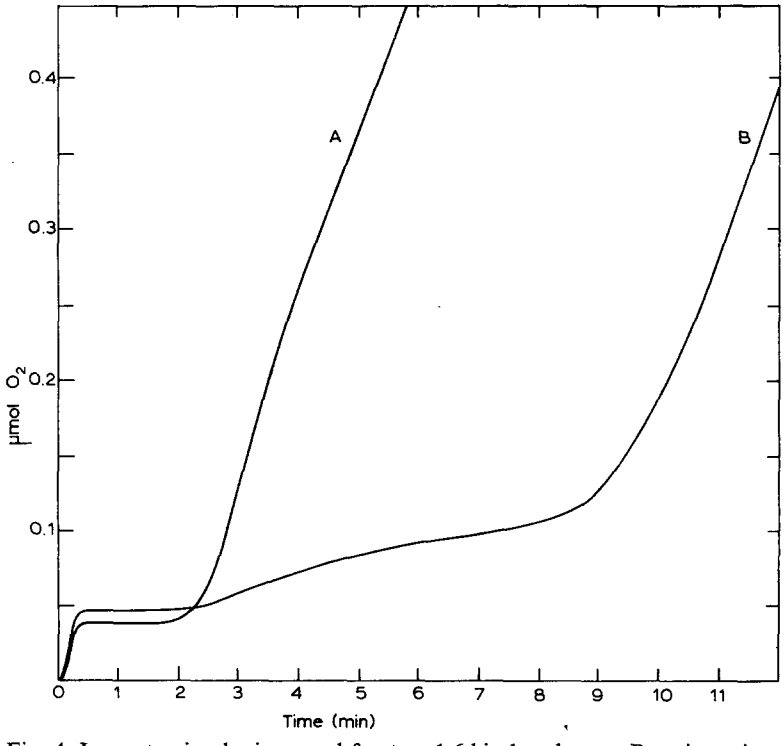

Fig. 4. Lag extension by increased fructose-1,6-bisphosphatase. Reaction mixtures standard except that each contained additional ferredoxin (180 µg in total) and chloroplast extract equivalent to only 100 µg chlorophyll. The chloroplast extract contained 0.189 units of fructose-1,6-bisphosphatase. Purified fructose-1,6-bisphosphatase (1 unit) was also added to B.

2. *Increased stromal protein.* Fig. 3 shows that the lag can be increased by increased stromal protein (chloroplast extract). The response is variable, depending upon the conditions used. The addition of such a complex mixture would be expected to influence a number of reactions. With Fru-1,6-P_2 as substrate, however, the increased fructose-1,6-bisphosphatase resulting from the presence of additional chloroplast extract might well be the most important single factor. This is borne out by the addition of purified fructose-1,6-bisphosphatase (Fig. 4) and Fru-6-*P* (Fig. 7).

3. *Increased fructose-1,6-bisphosphatase.* Like increased Fru-1,6-P_2, increased fructose-1,6-bisphosphatase can also bring about a marked extension of the lag (Fig. 4). It was to be expected, therefore, that similar lag extensions should follow changes in components which increase the fructose-1,6-bisphosphatase activity within the chloroplast extract (such as Mg^{2+}, pH, etc.).

4. *Increased pH.* With substrates other than Fru-1,6-P_2 near maximal activity was reached at pH 7.6. Fig. 5 shows that at this pH the lag was relatively short and that there was a considerable increase when the pH was raised to 7.9 (i.e. to that employed in all the other assays). A marked increase in chloroplast fructose-1,6-

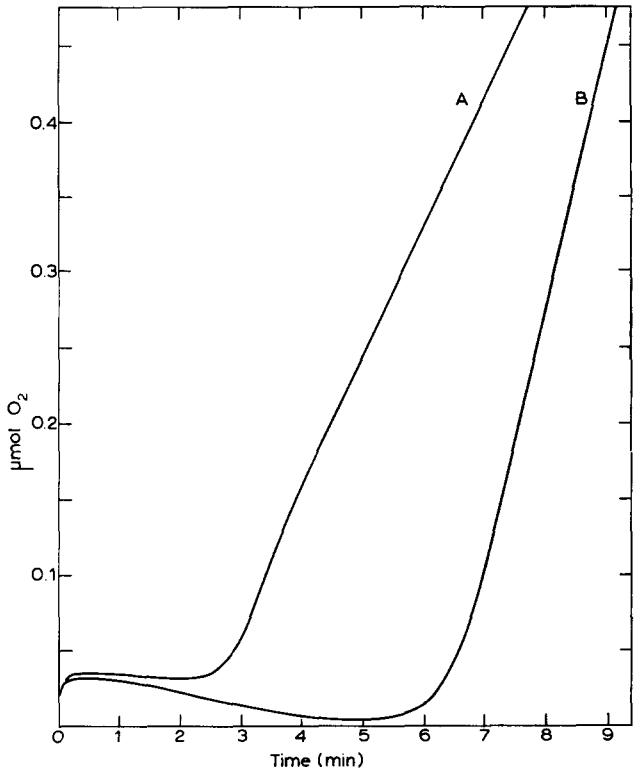

Fig. 5. Lag extension by increased pH. (A) pH 7.6 (B) pH 7.9. The mixtures were otherwise standard except that each contained 5 mM dithiothreitol. According to Baier and Latzko [18] a decrease in pH from 7.9 to 7.6 brings about a marked decrease in fructose-1,6-bisphosphatase activity. (It should be noted that in this experiment the Fru-1,6-P_2 (2 mM) was added 1 min prior to illumination and that this decreases the initial O_2 uptake, presumably as a result of reduction of NADP by glyceraldehyde 3-phosphate formed in the aldolase reaction.)

bisphosphatase activity over this pH range has been reported by Baier and Latzko [18].

5. *Increased* Mg^{2+}. With *sn*-phospho-3-glycerate as substrate, O_2 evolution proceeds rapidly in 1 mM $MgCl_2$ [9] but it is necessary to raise this in order to demonstrate CO_2-dependent O_2 evolution and the optimal concentration is nearer to 5 mM, depending on the conditions employed [9]. With Fru-1,6-P_2 as substrate a a further increase in Mg^{2+} is required and Fig. 6 shows that the lag was also extended as the [Mg^{2+}] was increased from 5 mM in C to 20 mM in D. Purified chloroplast fructose-1,6-bisphosphatase shows a similar increase in activity in the presence of high concentrations of Mg^{2+} [16, 18].

6. *Dithiothreitol.* Fructose-1,6-bisphosphatase is believed to be activated by several other factors including ferredoxin [16, 19]. All of the reaction mixtures used required ferredoxin to facilitate electron transport to NADP and the role of ferredoxin as an activator of fructose-1,6-bisphosphatase was not therefore specifically examined. On the other hand, activation by dithiothreitol can substitute for activation by ferredoxin [16, 18] and Fig. 6 shows the extent to which the lag was lengthened and the final rate of O_2 evolution increased as the dithiothreitol was increased from zero in B to 5 mM in D.

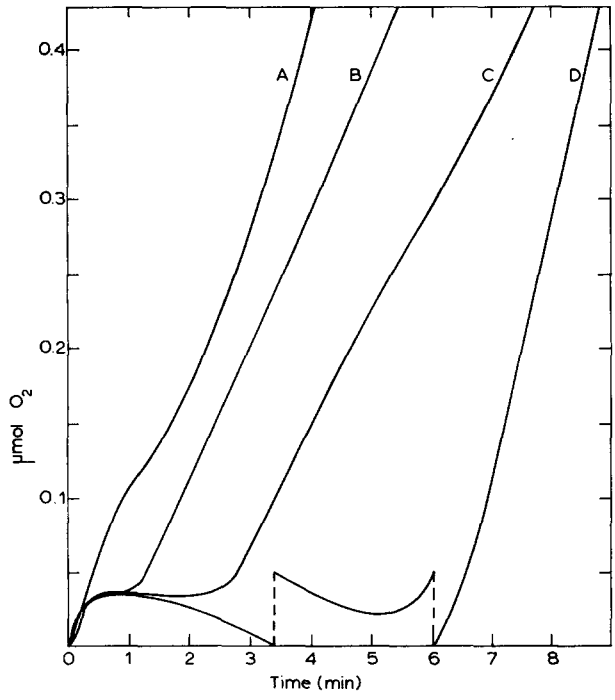

Fig. 6. Lag extension by increased Mg^{2+} (C vs. D), by increased dithiothreitol (B vs. D), and the elimination of the lag by addition of an ATP generator (A vs. D). (A) contained creatine phosphate (5 mM) and creatine phosphokinase (4 units), (B) contained no dithiothreitol and (C) contained only 5 mM $MgCl_2$ (rather than 20 mM as in the others). Mixtures otherwise standard except for the presence of 5 mM dithiothreitol in A, C and D. According to Baier and Latzko [18] both dithiothreitol and high Mg^{2+} are required for maximal fructose-1,6-bisphosphatase activity.

7. *Increased Fru-6-P.* All of the above factors are known, or believed, to increase the rate of conversion of Fru-1,6-P_2 to Fru-6-*P*. Ostensibly, direct substitution of Fru-6-*P* for Fru-1,6-P_2 is equivalent to an infinitely fast rate of conversion and as such should lead to maximal lag extension. This does not occur, but the short lag and relatively poor rate are readily explained by the fact that only traces of triose phosphate will be originally present in the mixture and the triose phosphate concentration can increase only by the operation of the full Benson-Calvin cycle. With Fru-1,6-P_2, on the other hand, the triose phosphate required for the transketolase reaction can also be provided by the action of aldolase. If this supposition is correct an equimolar mixture of Fru-1,6-P_2+Fru-6-*P* should give a longer lag than either Fru-1,6-P_2 or Fru-6-*P* alone and this is seen in Fig. 7. With Fru-1,6-P_2 alone the lag is limited by the rate of formation of Fru-6-*P*. With Fru-6-*P* alone the limiting factor is triose phosphate. With both hexose phosphates present the rate of formation of Rib-5-*P* is maximal and hence the lag is longer (Fig. 7).

It should be noted that in all of the above examples (except Fig. 2), lag extension is associated with an increased final rate. Again this is consistent with the fact that the longer lag is caused in each case by an increase in the Rib-5-*P* pool. Once this pool has been converted to Rbu-1,5-P_2 by photophosphorylation, then the rate of O_2 evolution will be limited only by the overall rate of turnover of the cycle and ultimately by electron transport. When the rate of Rib-5-*P* formation limits the size

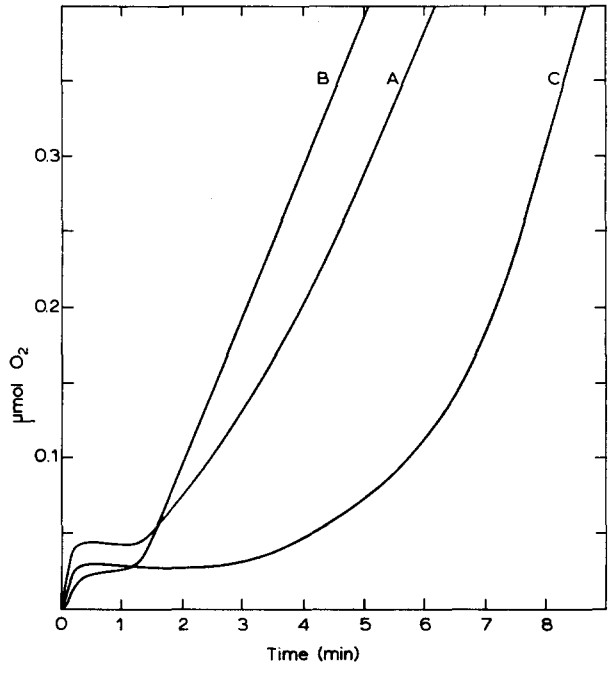

Fig. 7. Lag extension by increased Fru-6-*P*. (A) 2 mM Fru-6-*P*, (B) 2 mM Fru-1,6-P_2 and (C) 1 mM Fru-1,6-P_2+1 mM Fru-6-*P*. The effect of these additions (made immediately prior to illumination) is the same as instant conversion of 50 % of the Fru-1,6-P_2 to Fru-6-*P* (C) and 100 % of the Fru-1,6-P_2 to Fru-6-*P* in (B).

of the Rib-5-*P* pool it must also limit the rate of Rib-5-*P* utilisation so that both lag and final rate will be diminished.

Factors expected to affect the steady-state concentration of ADP

It has been seen in Fig. 1 that CO_2 fixation (and hence *sn*-phospho-3-glycerate formation) proceeds rapidly from the outset with Fru-1,6-P_2 as substrate so that the lag in O_2 evolution must be a consequence of some block on *sn*-phospho-3-glycerate reduction. If we are correct in attributing this to an unfavourable steady-state concentration of ADP the lag should be shortened by decreasing the steady state concentration of ADP and vice versa.

1. *Creatine phosphate plus creatine phosphate kinase.* The lag can be shortened, or eliminated at will, by the addition of creatine phosphate and creatine phosphate kinase (see, for example, Figs. 6A, 8A and 8C). Together, these two components constitute an ATP generator and, when they are present in relative excess, all of the reagents that normally extend the lag are without effect. Biphasic kinetics are often observed if creatine phosphate and creatine phosphate kinase are present from the outset (as in Fig. 6A), but not if there is appreciable pre-incubation with Fru-1,6-P_2. It seems probable that when the ATP limitation is removed other factors, such as the supply of triose phosphate, can become rate-limiting, but that this is less likely to occur if the Fru-1,6-P_2 is allowed to equilibrate with its products. Thus, in Fig. 6A the elimination of the initial lag is attributed to the correction of the adverse ATP/ADP ratio, the following decline to consumption of glyceraldehyde 3-phosphate derived from Fru-1,6-P_2 in the aldolase reaction and the subsequent increase to the renewed availability of glyceraldehyde 3-phosphate as it is formed by reduction of *sn*-phospho-3-glycerate.

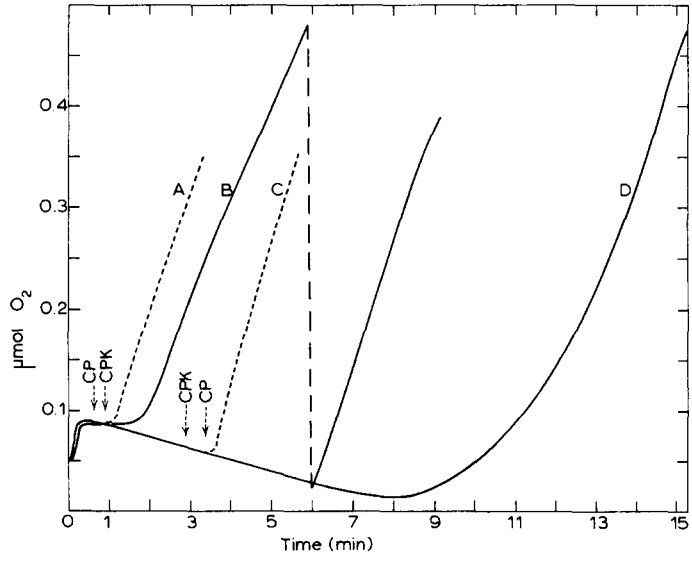

Fig. 8. Lag extension by antimycin A and its reversal by an ATP generator. Mixtures A, C and D contained 6 μM antimycin A in 3 μl of ethanol. B contained 3 μl of ethanol but no antimycin A. Creatine phosphate (CP) (5 mM) and creatine phosphokinase (CPK) (8 units) were added to A and to C, in reverse order, as indicated.

TABLE I

EFFECT OF FERREDOXIN CONCENTRATION ON LAG

Standard reaction mixtures contained 5 mM dithiothreitol, 2 mM Fru-1,6-P_2, and the quantities of ferredoxin indicated. The lag is taken as the time between the first cessation of O_2 evolution and the first detectable sign of its resumption (e.g. in Fig. 8D this interval would be put at 7.8 min).

Ferredoxin (μg/ml)	Lag (min)
270	0.8
180	1.8
90	8.2
45	9.5

It may be noted (Fig. 8) that neither creatine phosphate nor creatine phosphokinase produces any effect when added singly.

2. *Ferredoxin.* The length of the lag is progressively decreased by increases in the concentration of ferredoxin (Table I). Ferredoxin is a known catalyst of cyclic and pseudocyclic photophosphorylation and any ATP-requiring reactions which occur during the lag must be independent of noncyclic (O_2-evolving) phosphorylation unless this is masked by a simultaneous O_2 uptake. The reconstituted system contains sufficient catalase to preclude any net O_2 uptake consequent upon a Mehler reaction (i.e. pseudocyclic photophosphorylation resulting from reoxidation of reduced ferredoxin by molecular oxygen). The diminution of the lag by increased ferredoxin is therefore regarded as a consequence of increased cyclic photophosphorylation (cf. the effect of creatine phosphate plus creatine phosphokinase above). Activation of Fru-1,6-P_2 by ferredoxin [16, 19] would, of course, tend to lengthen the lag rather than shorten it, but it is assumed that in the presence of dithiothreitol such activation is largely complete [16] and that the contribution of ferredoxin to photophosphorylation predominates.

Antimycin A

This antibiotic is known to inhibit cyclic photophosphorylation catalysed by ferredoxin (see e.g. Arnon [20]). Fig. 8 shows that it acts as an antagonist of ferredoxin in the sense that it extends the lag whereas ferredoxin (Table I) diminishes the lag. Antimycin A may also activate fructose-1,6-bisphosphatase (Schacter and Bassham [21]) and if it does it would also promote a lag extension for this reason. Under the present conditions it would appear that it may affect the kinetics both by decreasing phosphorylation and by increasing the conversion of Fru-1,6-P_2 to Fru-6-P. Thus the lag may be entirely eliminated by creatine phosphate and its kinase but in the presence of antimycin A or dithiothreitol (a known activator of fructose-1,6-bisphosphatase), the rate is maintained at a high level (Table II), whereas in controls without these additives it soon declines. Similarly in Fig. 8 the final rates in the presence of antimycin A are faster than the control (B) whether the lag is allowed to terminate without intervention (D) or brought to an end by the addition of the ATP generator (A, C). These results show that there is a component of antimycin A action which can be simulated by dithiothreitol (cf. Fig. 6) and it could therefore be inferred that this component reflects increased fructose-1,6-bisphosphatase activity.

TABLE II

ABILITY OF DITHIOTHREITOL AND ANTIMYCIN A TO MAINTAIN OXYGEN EVOLUTION AT HIGH RATES

Standard reaction mixtures contained creatine phosphate (5 mM) +creatine phosphokinase (8 units) and either dithiothreitol (5 mM) or antimycin A (6 μM). The Fru-1,6-P_2 (2 mM) was added 3 min prior to illumination so that at first the reaction would not be limited either by Fru-6-*P* formation or availability of glyceraldehyde 3-phosphate. As the reaction procedes, however, the rate of Fru-6-*P* formation would be expected to become limiting and the slower decline in O_2 evolution in the presence of dithiothreitol is therefore in accord with the known activation of fructose-1,6-bisphosphatase by this reagent [16, 18]. The fact that antimycin A produces a very similar response to dithiothreitol is consistent with its postulated action as a fructose-1,6-bisphosphatase activator [21].

	O_2 evolved (μmol)		
Additive			
+Dithiothreitol	0.090	0.085	0.070
−Dithiothreitol	0.090	0.060	0.030
+Antimycin A	0.100	0.080	0.070
−Antimycin A	0.090	0.060	0.025
Time interval (min after start)	2–3	7–8	12–13

Interruption of O_2 evolution by the addition of ADP or ADP-generating systems

It is implicit in the preceding interpretation that the lag in O_2 evolution with Fru-1,6-P_2 as substrate is engendered, in the first place by the accumulation of Rib-5-*P* (which then acts as an ATP sink) and that it is terminated when this Rib-5-*P* pool is decreased to a size that allows the reduction of *sn*-phospho-3-glycerate to proceed at a measurable rate. With Rib-5-*P* and *sn*-phospho-3-glycerate as substrates O_2 evolution may be interrupted by the addition of ADP or any ADP-generating system such as glucose+hexokinase [12] Similar effects have been observed with Fru-1,6-P_2 as substrate. Particularly when the lag is long and CO_2 fixation indicates continuous *sn*-phospho-3-glycerate formation during the lag (as in Fig. 1) it is evident the final O_2 evolution is essentially *sn*-phospho-3-glycerate-dependent (rather than Fru-1,6-P_2-dependent) and might therefore be expected to respond in the same fashion.

DISCUSSION

In order to bring about CO_2 fixation and CO_2-dependent O_2 evolution Fru-1,6-P_2 must enter the "sugar phosphate shuffle" of the reductive pentose phosphate cycle (see e.g. Bassham and Calvin [22]). The first steps will involve the loss of phosphate from carbon-1 in a reaction catalysed by fructose-1,6-bisphosphatase, and presumably some direct production of triose phosphate in the aldolase reaction (Fig. 9). Pentose phosphate is then derived from Fru-6-*P* in the transketolase reaction and, in the presence of a continuing supply of ATP to phosphorylate Rbu-5-*P*, quantitative conversion to Rbu-1,5-P_2 would be expected. When ATP is continuously generated from ADP at the expense of creatine phosphate, CO_2 fixation and O_2 evolution both start without delay. When ATP production depends solely on photophosphorylation, there is a lag in O_2 evolution which may be increased by increasing the Fru-1,6-P_2 concentration. With Rib-5-*P* the lag is greater and it has been concluded that the

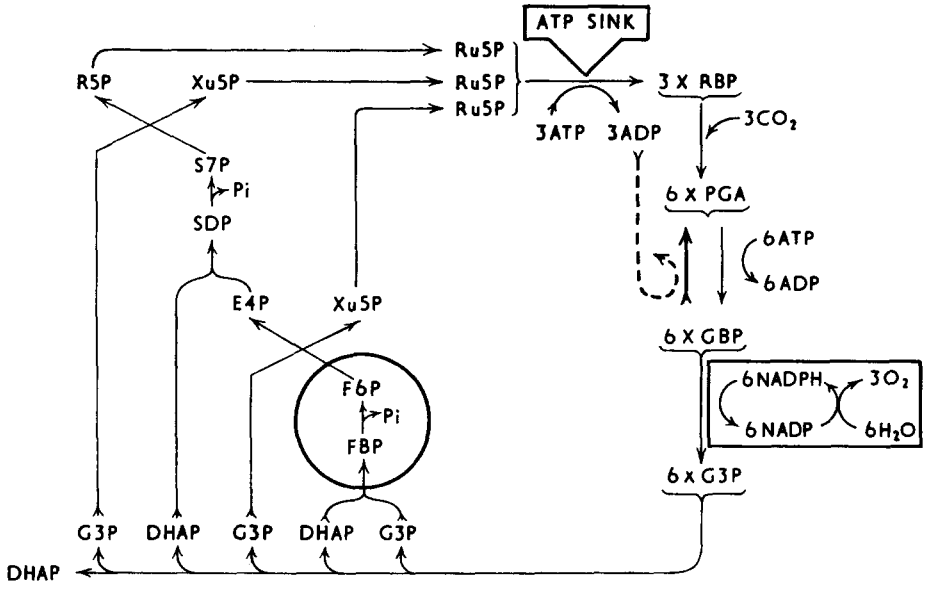

Fig. 9. The events which bring about the lag in O_2 evolution with Fru-1,6-P_2 as substrate and influence its extent. The diagram outlines the reactions of the reductive pentose phosphate cycle. When Fru-1,6-P_2 (FBP) is introduced in substrate quantities (circle, bottom left) it initiates a series of reactions. CO_2 fixation starts very quickly, indicating that *sn*-phospho-3-glycerate (PGA) is being formed. O_2 evolution (apart from that associated with the reduction of catalytic NADP) is delayed showing that *sn*-glycerol 1,3-bisphosphate (GBP) is not being reduced to glyceraldehyde 3-phosphate (G3P). If it were, NADPH would be reoxidised to NADP and this would accept electrons from water (via the photosynthetic electron transport sequence) and O_2 would be evolved (see box at bottom right). The reason that O_2 evolution is delayed despite the availability of *sn*-phospho-3-glycerate, ATP and NADPH is that Rbu-5-*P* (Ru5P) (top centre) formed from Fru-1,6-P_2 acts as a highly efficient sink for ATP, thus regenerating ADP as rapidly as endogenous photosynthetic phosphorylation can form ATP. The steady-state concentration of ADP is therefore high and (heavy arrow) favours the conversion of *sn*-glycerol 1,3-bisphosphate to *sn*-phospho-3-glycerate rather than its reduction [12]. Eventually, however, the newly generated Rbu-5-*P* pool shrinks as the Fru-1,6-P_2 from which it is derived shrinks, the ADP concentration falls, *sn*-glycerol 1,3-bisphosphate is reduced and O_2 is evolved. Factors which favour the conversion of Fru-1,6-P_2 to Fru-6-*P*(F6P) lead to the formation of a large Rbu-5-*P* pool and a long lag. Factors which favour the conversion of ADP to ATP shorten the lag.

advent of O_2 evolution is delayed until the concentration of Rbu-5-*P* in the system (or the rate of its generation from Rib-5-*P*) no longer has a sufficient adverse effect on the ADP/ATP ratio to inhibit the conversion of glyceraldehyde 3-phosphate to *sn*-glycerate 1,3-bisphosphate [12]. Without this conversion NADPH reoxidation cannot proceed and accordingly O_2 evolution is inhibited. The present results indicate that (with Fru-1,6-P_2) a steady state is soon established so that in many circumstances tha rate of formation of Rbu-5-*P* is not too fast to inhibit O_2 evolution completely. This would explain the biphasic kinetics often observed (see, for example, Fig. 6A) in which the second acceleration would mark the end of the drain on ATP engendered by Rbu-5-*P* derived from Fru-1,6-P_2. If this conclusion is correct, any factor which materially accelerated fructose-1,6-bisphosphatase activity would be expected to increase the lag. This proposal is, therefore, considerably strengthened by the fact that

the lag is markedly lengthened by the presence of dithiothreitol, high magnesium and high pH, all of which are known to activate fructose-1,6-bisphosphatase [16, 18]. Moreover, the lag is also extended by the addition of a purified fructose-1,6-bisphosphatase (Fig. 4) or the end product of the reaction which it catalyses (Fig. 7). Conversely, the lag is abolished by the addition of an ATP-generating system (Figs. 6 and 8). It is also shortened by increasing the ferredoxin concentration (Table I) which, it is suggested, in turn increases the rate of cyclic or pseudocyclic photophosphorylation which must proceed during the lag.

The role of antimycin A in these events could be explained in two ways. If antimycin A activated fructose-1,6-bisphosphatase as proposed by Schacter and Bassham [21] there is no doubt that it would extend the lag (cf. Fig. 4). Alternatively, if it inhibited ATP formation to a small extent it would produce the same effect. At the concentrations used, antimycin A is without effect on electron transport to NADP and its effect on photosynthetic control is negligible. On the other hand, it slows *sn*-phospho-3-glycerate-dependent O_2 evolution and extends the interruption of *sn*-phospho-3-glycerate-dependent O_2 evolution by glucose and hexokinase when these are used to simulate the drain on ATP brought about by the addition of Rib-5-*P* (Fitzgerald and Walker, unpublished). Clearly these effects cannot be attributed to an activation of fructose-1,6-bisphosphatase. However, when constraints on ATP formation are removed by the addition of creatine phosphate and creatine phosphokinase (and the Fru-1,6-P_2 lag therefore eliminated) the rate of O_2 evolution in the presence of antimycin A is faster than in its absence (Fig. 8 and Table II). Accordingly we must conclude that antimycin A produces its response by affecting both fructose-1,6-bisphosphatase and photophosphorylation. Since antimycin A is without effect in the spectrophotometric assay of fructose-1,6-bisphosphatase (Slabas and Walker, unpublished) it seems probable that it produces its effect indirectly by affecting the reduction status of ferredoxin, which has been shown to activate fructose-1,6-bisphosphatase [16, 19]. It has been concluded that antimycin A can affect electron transport through photosynthetic cytochromes and thus inhibit cyclic photophosphorylation [20]. Also the activation of fructose -1,6-bisphosphatase by ferredoxin has been attributed to the reduction of sulphydryl groups [16]. If antimycin A interferes with the normal course of reoxidation of reduced ferredoxin, it is conceivable that its action at a single site could simultaneously slow ATP formation from ADP and hasten Fru-6-*P* formation from Fru-1,6-P_2. In turn, both of these effects would lower the steady-state concentration of ADP and hence retard *sn*-phospho-3-glycerate reduction and its associated O_2 evolution. The consequences of these effects bear upon the many observations relating to the impact of antimycin A on photosynthesis by intact chloroplasts [23–28].

It is generally accepted that in photosynthesis one molecule of O_2 is evolved for every molecule of CO_2 fixed and if CO_2 is to be reduced to the level of carbohydrate at the expense of water there is no possibility that this stoichiometry can be avoided in anything other than transient changes. There are, however, at least three branching points in photosynthetic carbon metabolism and it would be of interest to know what determines how much traffic is directed along each route. The three major pathways are as follows. (1) Newly fixed carbon can be retained within the cycle, (2) it can be retained within the chloroplast but diverted into a variety of end-products such as starch, or (3) it may be released to the cytoplasm. The present results do not

explain the problems of regulation that the existence of these three pathways raises, but they do show how a perturbation at one point in the Benson-Calvin cycle can influence the course of distant reactions. In many circumstances it may be supposed that the triose phosphate that remains in the chloroplast enters the transketolase and aldolase reactions in the proportions of two to three and this is certainly a prerequisite if maximal regeneration of pentose phosphate is to be achieved. These proportions will presumably be largely self-regulatory. Thus, if Fru-6-*P* were in relative deficit, the first transketolase reaction would be limited, triose phosphate would be diverted into the aldolase reaction and Fru-1,6-P_2 would become available as a source of Fru-6-*P* (Fig. 10A). Conversely, if Fru-6-*P* were in excess it would divert triose phosphate from the aldol condensation and thereby decrease its own production (Fig. 10B). The effects of stimulated changes in the rate of conversion of Fru-6-*P* to Fru-1,6-P_2 are seen in Fig. 7. Neither Fru-6-*P* nor Fru-1,6-P_2 alone can yield Rbu-5-*P* at a sufficient rate to bring about a prolonged retardation of O_2 evolution, whereas if both are supplied together in the same total quantity the lag is extended. Similarly the activation of fructose-1,6-bisphosphatase will prolong the lag whereas total conversion to Fru-6-*P* (i.e. application as Fru-6-*P* alone) will not, because in these circumstances further metabolism is diminished by the relative deficiency of triose phosphate. It is suggested that these facts may bear on the control of starch formation and on the proportions of photosynthetic product which follow the three routes listed above. During induction (i.e. immediately after passing from prolonged darkness into bright light) the availability of ATP and NADPH will be less likely to limit photosynthesis than the availability of metabolites of the carbon cycle. There will be little tendency for *sn*-phospho-3-glycerol to accumulate or for triose phosphate to be lost from the chloroplast. Instead, the pull provided by the ATP- and NADPH-consuming reactions will favour retention and circulation within the cycle and therefore permit the autocatalytic acceleration, which is a characteristic feature of induction. In the steady state, however, reduction will be limited by the supply of ATP and NADPH and the pool of *sn*-phospho-3-glycerate will tend to increase. Similarly, lack of ATP will favour pentose monophosphate accumulation (see, for example, Gibbs [28]) and the back pressure of pentose monophosphates in the transketolase reaction will divert Fru-6-*P* towards starch formation. When the pentose phosphate pool is full the availability of triose phosphate for export and starch synthesis will

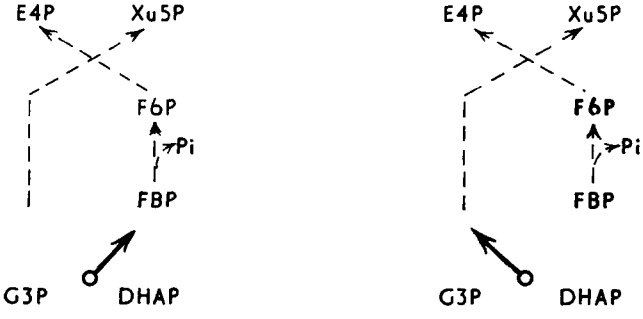

Fig. 10. Diversion of triose phosphate towards consumption in aldolase or transketolase reaction. (A) Low Fru-6-*P* (F6P) will favour consumption by aldolase. (B) High Fru-6-*P* will favour consumption by transketolase. Normally this process will be self-regulating but obviously it would respond to natural or artificial activators of fructose-1,6-bisphosphatase such as dithiothreitol.

be maximal and the activity of fructose-1,6-bisphosphatase will be one of a number of factors that will influence which of these two routes predominates. Ultimately pentose phosphate must also limit its own production because if ATP consumption in the ribulose kinase reaction becomes excessive, the reduction of *sn*-phospho-3-glycerate will cease and the pentose phosphate pool will diminish accordingly.

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* Publisher's note: in this article (Transient inhibition by ribose 5-phosphate of photosynthetic O_2 evolution in a reconstituted chloroplast system, by A.R. Slabas and D. A. Walker), an error occurred owing to the introduction of new nomenclature. *sn*-Phospho-3 glycerol and *sn*-glycerol-1,3-bisphosphate should read *sn*-phospho-3-glycerate and *sn*-glycerate-1,3-bisphosphate, respectively, throughout the entire text. (Formerly, *sn*-phospho-3-glycerate was referred to as 3-phosphoglycerate and *sn*-glycerate-1,3-bisphosphate was referred to as 1,3-diphosphoglycerate or glycerate-1,3-bisphosphate).