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SOME EFFECTS OF INORGANIC PHOSPHATE ON O_2 EVOLUTION BY ISOLATED CHLOROPLASTS

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SUMMARY

1. After an initial lag, isolated spinach chloroplasts evolved O_2 in illuminated reaction mixtures containing bicarbonate but no added phosphate. This evolution soon ceased but could be restarted by the addition of phosphate.

2. The phosphate requirement could be met by orthophosphate, inorganic pyrophosphate, ATP or ADP but not by AMP. Approx. 3 molecules of O_2 were evolved for each molecule of orthophosphate added and approx. 6 for each molecule of pyrophosphate.

3. With CO_2 as the sole added substrate the extent of the initial lag in O_2 evolution was not greatly affected by small quantities of added orthophosphate but as the concentration of orthophosphate was increased there was a progressive increase in the lag and a progressive decrease in the maximum rate. Pyrophosphate failed to produce these effects at a 100 times the concentration and in the presence of pyrophosphate the orthophosphate inhibition was less severe. There was little or no orthophosphate inhibition in the presence of substrate quantities of 3-phosphoglycerate or ribose 5-phosphate and CO_2.

4. There was also a requirement for phosphate by chloroplasts evolving O_2 in the presence of 3-phosphoglycerate or ribose 5-phosphate *plus* CO_2. In the presence of endogenous phosphate only, added ribose 5-phosphate suppressed the O_2 evolution which normally followed the addition of 3-phosphoglycerate.

5. The results provide direct support for the proposed phosphate requirement of the photosynthetic carbon cycle and are discussed in this context. They also imply that orthophosphate, ribose 5-phosphate and 3-phosphoglycerate can penetrate the intact chloroplast envelope with considerable rapidity.

INTRODUCTION

It is known that CO_2 fixation by isolated chloroplasts is stimulated by low concentrations of orthophosphate and inhibited by high concentrations (see *e.g.* refs. 1, 2). JENSEN AND BASSHAM[3] have also reported a stimulation by inorganic pyrophosphate. Our own experience has led us to conclude that the concentration of

Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate.

orthophosphate in reaction mixtures can bring about striking changes in the kinetics of CO_2 fixation and O_2 evolution[4,5]. Such effects are of interest because of their possible relationship to metabolic control mechanisms and for these reasons a more complete investigation seemed desirable.

MATERIALS AND METHODS

Chloroplast isolation

Chloroplasts were isolated from spinach (grown in the Chelsea Physic Garden or purchased in the local market) using methods described previously[5]. The solutions used were essentially similar to those used by JENSEN AND BASSHAM[3] except that $NaNO_3$ and KH_2PO_4 were not constituents of the isolating and resuspending media. The isolating medium contained sorbitol, 0.33 M; MES (2-(*N*-morpholino)ethanesulphonic acid)–NaOH (pH 6.1), 0.05 M; NaCl, 20 mM; sodium isoascorbate, 2 mM; $MnCl_2$, 1 mM; and $MgCl_2$, 1 mM. The resuspending solution was similar to the isolating medium except that it was buffered to pH 6.7 with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid)–NaOH. The basic incubation medium was again similar except that it did not contain NaCl and was buffered to pH 7.6 with 0.05 M HEPES–NaOH. Reaction mixtures contained 1.6 ml of incubation medium to which were added 0.2 ml resuspending medium containing 162 μg chlorophyll. The final volume of 1.8 ml included where appropriate Na_2CO_3 (18 μmoles), orthophosphate, pyrophosphate, ATP, ADP, AMP, ribose 5-phosphate (12 μmoles), 3-phosphoglycerate (12 μmoles).

Envelope-free chloroplasts

In the experiment relating to Fig. 10 chloroplasts were caused to shed their outer envelopes by adding the chloroplast suspension to an incubation medium from which the osmoticum (sorbitol) was absent. After exposure to these hypotonic conditions (< 0.04 M) for 1 min the sorbitol was added. Thus final reaction mixtures containing either 'whole' or envelope-free chloroplasts were identical—except in relation to the organisation of the plastids.

Measurement of O_2 evolution

In experiments relating to Figs. 3–6, 8 and 9 O_2 evolution was measured using a Rank oxygen-electrode cell operated and illuminated as detailed previously[5]. In later experiments relating to Figs. 1, 2, 7 and 10 two separate Rank cells were used in conjunction with a twin channel recorder. This allowed two assays to be carried out simultaneously and diminished problems associated with chloroplast deterioration.

RESULTS

The effect of phosphate on O_2 evolution with CO_2 as the sole added substrate

In the absence of added orthophosphate (and without added substrate other than CO_2) illuminated chloroplasts started to evolve O_2 after an initial lag (*cf.* refs. 4–6). If the chloroplasts had been prepared in phosphate-free medium the evolution soon ceased but could then be restarted (see *e.g.* Fig. 3) by the addition of orthophosphate or inorganic pyrophosphate[4]. The O_2 evolved was then quantitatively related to the added phosphate. The addition of a small quantity of orthophosphate was

followed by the evolution of approx. 3 molecular equivalents of O_2 (ref. 4). The same response was induced by half the quantity of pyrophosphate[4]. Fig. 1 shows that the requirement for phosphate could also be met by ATP or ADP. There was no response to the addition of AMP. The kinetics of O_2 evolution following the addition of orthophosphate were closely similar to those following the addition of pyrophosphate except that the first recorded response to pyrophosphate occurred some 20 sec after the corresponding response to orthophosphate[4]. Fig. 2 shows that the kinetic response to ATP was somewhat different, with a much slower response and a slower rate of evolution.

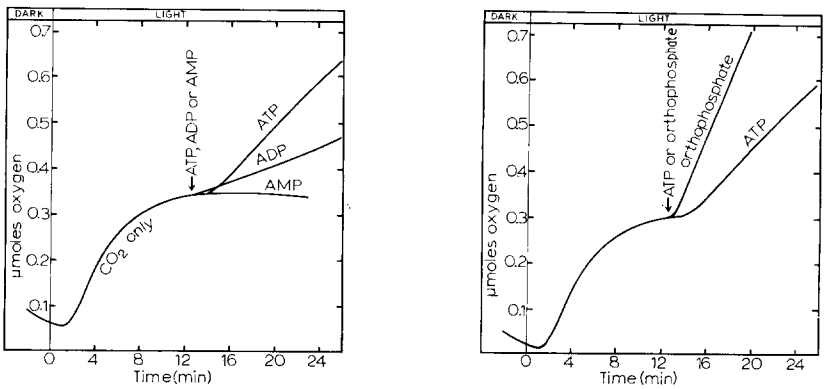


Fig. 1. Restoration of O_2 evolution by ATP in the absence of exogenous orthophosphate. Chloroplasts were prepared, resuspended and incubated in media from which inorganic phosphate was omitted. Bicarbonate (18 μmoles) was present and a short-lived O_2 evolution followed illumination (*cf.* ref. 4). When net evolution had ceased, 1 μmole of AMP, ADP or ATP was added as indicated.

Fig. 2. The kinetics of O_2 evolution following the addition of ATP or orthophosphate. Experimental conditions as for Fig. 1. When net evolution had ceased 0.25 μmole of orthophosphate or 1 μmole of ATP were added as indicated.

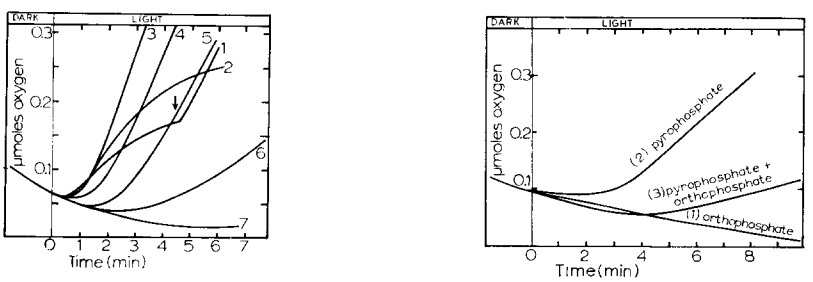


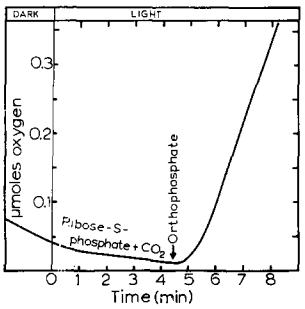
Fig. 3. The relationship between orthophosphate concentration and O_2 evolution with CO_2 as the sole added substrate (*cf.* Fig. 6). Chloroplasts were prepared and resuspended in media from which inorganic phosphate was omitted. In addition to bicarbonate (see METHODS) the reaction mixtures contained the following quantities of orthophosphate (in μmoles): (1) none, (2) 0.03, (3) 0.10, (4) 0.45, (5) 0.90, (6) 1.8, (7) 2.7. In (1), additional orthophosphate (0.9 μmole in 9 μl) added as indicated. The inhibition (Curves 4–7) was not produced if inorganic pyrophosphate was substituted for orthophosphate (see text).

Fig. 4. Alleviation of orthophosphate inhibition by inorganic pyrophosphate. Reaction mixtures contained CO_2 (18 μmoles bicarbonate) as the sole added substrate and (in Curve 1) sufficient orthophosphate (5 μmoles) to inhibit O_2 evolution (*cf.* Fig. 3). If inorganic pyrophosphate (50 μmoles) was substituted for orthophosphate (2) there was much less inhibition. Added together (3) the mixture of orthophosphate and pyrophosphate gave an intermediate effect.

The effect on the kinetics of O_2 evolution, of increasing orthophosphate concentration is given in Fig. 3. The onset of evolution in the presence of exogenous phosphate was not appreciably hastened by additions of orthophosphate at concentrations which prolonged O_2 evolution (Fig. 3, Curves 1–3). Instead, above a final concentration of about $5 \cdot 10^{-5}$ M further additions of orthophosphate brought about a progressive extension of the lag and depression of the maximum rate (Fig. 3, Curves 4–7). At low concentrations, pyrophosphate behaved like orthophosphate but at higher concentrations there was no appreciable inhibition until a very much higher concentration (about $5 \cdot 10^{-3}$ M) was reached. Moreover, when added together with an excess of pyrophosphate the inhibitory effect of orthophosphate was considerably less severe (Fig. 4).

The effect of phosphate on O_2 evolution in the presence of substrate concentrations of ribose 5-phosphate

The short-lived O_2 evolution which was seen in the absence of added orthophosphate (above) was depressed by the presence of substrate quantities of ribose 5-phosphate (Fig. 5). This effect could only be seen with ribose 5-phosphate samples which were largely free of contaminating orthophosphate. (The effective concentrations were such that there was a marked difference in response to samples of ribose 5-phosphate containing 3 % and 0.6 % orthophosphate, respectively.) It was more pronounced with some chloroplast preparations than others and it is assumed that it was most clear when the concentration of endogenous phosphate was lowest. This inhibitory effect could be rapidly relieved by the addition of orthophosphate (Fig. 5) or pyrophosphate. The effect of increasing orthophosphate concentration is seen in Fig. 6 and it should be noted that this is the converse of that seen in Fig. 3, *i.e.* within the concentration range shown, increases in the added orthophosphate brought about a progressive decrease in the time which elapsed before the attainment of the



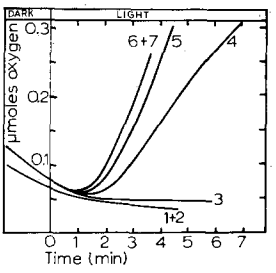


Fig. 5. Suppression, by ribose 5-phosphate, of the short-lived O_2 evolution which occurred in the presence of CO_2 and absence of added inorganic phosphate (*cf.* Figs. 1 and 2). Chloroplasts were prepared, resuspended and incubated in media (see METHODS) from which inorganic phosphate was omitted. The reaction mixture also contained 12 μmoles of ribose 5-phosphate. Orthophosphate (0.9 μmole in 9 μl) was added at the time indicated. O_2 evolution could also be restored by the addition of inorganic pyrophosphate to a similar concentration.

Fig. 6. The relationship between orthophosphate concentration and O_2 evolution in the presence of substrate concentrations of ribose 5-phosphate. The conditions were the same as those described in the legend to Fig. 3 except that in addition each reaction mixture contained ribose 5-phosphate (12 μmoles). It should be noted that, under these conditions, increasing phosphate concentration brought about a progressive decrease in the lag and increase in the rate of evolution (*cf.* the converse effect in Fig. 3). The effect of inorganic pyrophosphate was similar to that of orthophosphate.

maximum rate. Pyrophosphate produced essentially similar results. The inhibition by higher concentrations of orthophosphate seen with CO_2 as the only added substrate (above) was not recorded when substrate concentrations of ribose 5-phosphate were also present (*cf.* Figs. 3 and 6).

The effect of phosphate on O_2 evolution in the presence of substrate concentrations of 3-phosphoglycerate

In the preceding section it was reported that the short-lived O_2 evolution seen in the presence of CO_2 and endogenous phosphate[4] (see Figs. 1 and 2) was depressed by the addition of substrate concentrations of ribose 5-phosphate (Fig. 5). The addition of 3-phosphoglycerate had the converse effect, prolonging O_2 evolution (Fig. 7) although at a lower rate than that observed in the presence of added orthophosphate. If, however, substrate concentrations of ribose 5-phosphate were added together with the 3-phosphoglycerate the O_2 evolution was again depressed and the depression could again be relieved by the addition of orthophosphate or pyrophosphate (Fig. 8).

If chloroplasts were illuminated in the presence of CO_2 (but without added phosphate) until net evolution ceased (*cf.* Figs. 1 and 2) the addition of substrate concentrations of 3-phosphoglycerate did not bring about renewed O_2 evolution unless orthophosphate or pyrophosphate were also added (Fig. 7, Curve 4). The addition of orthophosphate at this stage might have been expected to bring about O_2 evolution irrespective of the presence of 3-phosphoglycerate (*cf.* Figs. 1 and 2). However, at higher concentrations orthophosphate was inhibitory in mixtures containing CO_2

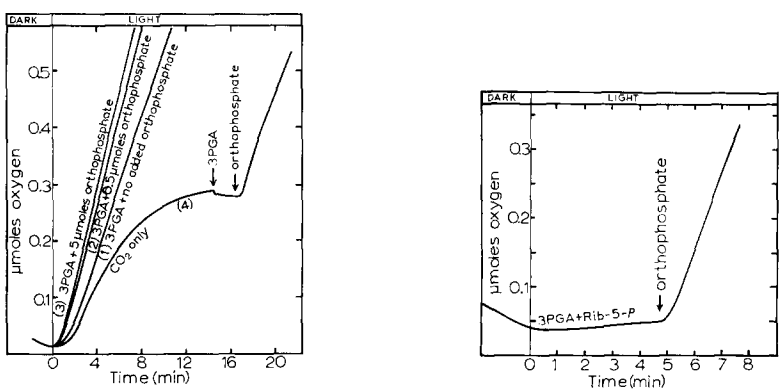


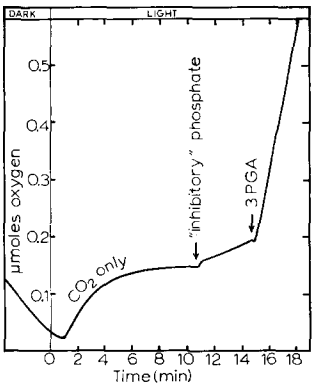
Fig. 7. The effect of orthophosphate on O_2 evolution in the presence of added 3-phosphoglycerate (3PGA). Chloroplasts were prepared and resuspended in media from which inorganic phosphate was omitted. Reaction mixtures (1, 2 and 3) contained 3-phosphoglycerate (12 µmoles) and in addition orthophosphate as follows (in µmoles): Curve 1, none; Curve 2, 0.5; Curve 3, 5. In (1) O_2 evolution was still continuing at a rate of 9 µmoles/mg chlorophyll per h after 36 min by which time a total of 1.35 µmoles had been evolved. In (4) the 3-phosphoglycerate was initially replaced by bicarbonate and added, as indicated when net O_2 evolution had ceased. No further O_2 evolution occurred until the further addition of orthophosphate (5 µmoles).

Fig. 8. Suppression, by ribose 5-phosphate, of the O_2 evolution which occurred in the presence of added 3-phosphoglycerate (3PGA) and absence of inorganic phosphate (*cf.* Figs. 5 and 7). Chloroplasts were prepared, resuspended and incubated in media (see METHODS) from which inorganic phosphate was omitted. The reaction mixtures also contain ribose 5-phosphate (12 µmoles) and 3-phosphoglycerate (12 µmoles). Orthophosphate (0.9 µmole in 9 µl) was added at the time indicated. Evolution could also be restored by the addition of pyrophosphate to a similar concentration.

alone (Fig. 3) and it can be seen in Fig. 9 that its addition at this concentration had little effect on O_2 evolution prior to the subsequent addition of 3-phosphoglycerate.

Permeability of chloroplast envelopes

Between 50 % and 90 % of chloroplasts isolated in sugar media may retain their envelopes[7]. After brief osmotic shock envelopes can no longer be seen. This treatment strongly depresses CO_2 fixation but it increases the photophosphorylation of exogenous ADP in the presence of pyocyanine[7]. It also increases the rate of O_2 evolution in the presence of ferricyanide and in mixtures containing $NADP^+$ and ferredoxin (Table I), presumably by removing barriers between these additives and the active sites within the chloroplasts. While it is accepted that disrupted chloroplasts can evolve O_2 an adverse effect of osmotic shock was to be expected in systems utilising a product of CO_2 fixation as the hydrogen acceptor (*cf.* ref. 7). This was borne out by the experiment illustrated in Fig. 10 in which osmotic shock (achieved simply by altering the order in which additions were made to the reaction mixtures) almost abolished O_2 evolution. Although osmotic shock undoubtedly affects the chloroplasts in several ways, all of the above results are consistent with the view that the intact envelope prevents enzyme loss and, conversely, limits the penetration of some external molecules. If it is accepted that the intact envelope is necessary for O_2 evolution in the presence of 3-phosphoglycerate or ribose 5-phosphate *plus* CO_2 (Fig. 10) then the present results also have some bearing on the rates at which certain



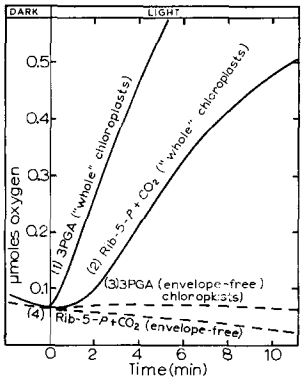


Fig. 9. O_2 evolution in the presence of added 3-phosphoglycerate (3PGA), a requirement for phosphate. Chloroplasts were prepared, resuspended and incubated in media from which inorganic phosphate was omitted. Bicarbonate was added at the outset and a short-lived O_2 evolution was observed (*cf.* Figs. 1 and 2). When net evolution had ceased orthophosphate (2.5 μmoles in 25 μl) was added but O_2 was evolved only slowly until the further addition of 3-phosphoglycerate (10 μmoles in 100 μl) (*cf.* Fig. 7, Curve 4). It should be noted that the concentration of orthophosphate added was sufficient to inhibit most of the O_2 evolution seen in the presence of CO_2 as the sole added substrate (Fig. 3), but insufficient to depress that in the presence of 3-phosphoglycerate (Fig. 7, Curve 3).

Fig. 10. The effect of osmotic shock on O_2 evolution by isolated chloroplasts. In addition to incubating medium, reaction mixtures 2 and 4 contained ribose 5-phosphate (12 μmoles) *plus* CO_2 (18 μmoles bicarbonate); 1 and 3 contained 3-phosphoglycerate (3PGA) (12 μmoles). In 1 and 2 the chloroplasts were added to otherwise complete reaction mixtures so that they were not exposed to osmotic shock. In 3 and 4 this normal procedure was varied so that the chloroplasts were briefly (1 min) exposed to a hypotonic medium (less than 0.04 M with respect to sorbitol) before the addition of the incubating medium.

TABLE I

O_2 EVOLUTION ASSOCIATED WITH THE REDUCTION OF $NADP^+$ BY 'WHOLE' AND OSMOTICALLY SHOCKED CHLOROPLASTS

These previously unpublished results were obtained during the course of work by WALKER AND HILL[6] and the chloroplasts were prepared and assayed accordingly. Reaction mixtures (5 ml) contained spinach chloroplasts (0.1 ml, approx. 0.1 mg chlorophyll), ferredoxin (0.35 mg), $NADP^+$ (3 μmoles), tricine–NaOH (0.04 M, pH 7.4), resuspending medium (3 ml) and breaking medium (1.5 ml). The resuspending medium contained 30.8 g sucrose, 200 mg NaCl, 200 mg $MgCl_2$ and 216 mg sodium isoascorbate in 200 ml of water. The breaking medium was the same as the resuspending medium except that the sucrose was omitted. Osmotic shock was achieved in the reaction mixtures by adding chloroplasts to the breaking medium before adding resuspending medium. In reaction mixtures containing 'whole' chloroplasts the order of additions was changed (breaking medium, followed by resuspending medium followed by chloroplasts). Somewhat similar increases in rate following osmotic shock were observed when $K_3Fe(CN)_6$ was substituted for ferredoxin and $NADP^+$.

Expt. No.	*Maximal rate of O_2 evolution (μmoles/h)*	
	Whole	*Osmotically shocked*
1	0.45	2.25
2	0.30	1.97

compounds penetrate the bounding membranes. Allowing for the response time of the instrument (about 2 sec) the penetration of orthophosphate (see *e.g.* Figs. 2, 3 and 8 and ref. 4) was extremely rapid and comparable to the response to added bicarbonate[6]. Similarly 3-phosphoglycerate caused an abrupt change in O_2 evolution within 20 sec of its addition (Fig. 9). Comparable results were obtained when ribose 5-phosphate was added to mixtures containing CO_2 and high concentrations of orthophosphate (as for Fig. 5, but orthophosphate added prior to ribose 5-phosphate). In contrast ATP and ADP appear to penetrate more slowly (Figs. 1 and 2) but since the responses could clearly be brought about by external hydrolysis and penetration of resulting orthophosphate the kinetics may reflect not the rate of penetration of ATP or ADP but the rate of their external hydrolysis.

Variability in chloroplast preparations

It should be noted that the kinetic responses obtained under otherwise standard conditions may vary according to the nature and previous history of the parent tissue[2,6]. Clearly, the concentrations of various metabolites in a freshly gathered preilluminated leaf may be quite different from those in a leaf which has been stored in a dark, cold room for a number of weeks. Quite considerable differences may be induced by the addition of certain cycle intermediates[8,9] at $5 \cdot 10^{-4}$ M and by orthophosphate at $5 \cdot 10^{-5}$ M (Fig. 3). Variations in the endogenous concentration of these compounds might easily be of this order of magnitude. In Figs. 1 and 2, if it is assumed that the initial O_2 evolution proceeded at the expense of endogenous phosphate with the same stoichiometry as that previously observed (3 O_2 per 1 phosphate), the original amount of utilizable phosphate would be approximately equivalent to 0.05 μmole of orthophosphate. It will be seen from Fig. 3 that if this level had been double the quantitative difference in kinetics would have been appreciable.

DISCUSSION

Isolated chloroplasts will evolve O_2 when illuminated in the presence of CO_2 as the sole added substrate[6,10]. The present results (see also ref. 4) show that sustained evolution was dependent on added phosphate. In the absence of exogenous phosphate, evolution soon ceased but could be restarted by the addition of phosphate. The amount of O_2 then evolved was quantitatively related to the phosphate added in proportions consistent with the synthesis of 1 molecule of triose phosphate for every 3 molecules of O_2 produced[4]. Inorganic pyrophosphate promoted twice as much O_2 evolution as equimolar orthophosphate[4]. The kinetics of O_2 evolution following the addition of orthophosphate and pyrophosphate were very similar except that with pyrophosphate there was an additional small delay of about 20 sec before the response was seen[4]. If 1 molecule of pyrophosphate produces the same effect as 2 molecules of orthophosphate the likelihood of hydrolysis cannot be readily discounted. However, for two reasons, we were inclined to suspect that pyrophosphate might be directly consumed in photophosphorylation (it is possible to write several plausible theoretical schemes which would account for the observed stoichiometry). The first reason was the close similarity of the kinetics, implying that, if hydrolysis occurred, it must be rapid and complete. The second was the failure of pyrophosphate to inhibit at concentrations 100-fold in excess of an amount of orthophosphate which produced a marked inhibition in similar reaction mixtures (Fig. 3). This seemed incompatible with any appreciable hydrolysis of pyrophosphate which would soon lead to the formation of inhibitory levels of orthophosphate. This argument was subsequently weakened by the surprising finding that orthophosphate was less inhibitory in the presence of excess pyrophosphate. The question of direct participation of pyrophosphate in photophosphorylation by chloroplasts must therefore remain open (*cf.* ref. 11) but whatever the mechanism, the present results clearly show that chloroplasts can utilise pyrophosphate as readily as orthophosphate. *In vivo*, a large proportion of the inorganic phosphate available in photosynthesis may well be in the form of pyrophosphate released in starch and sucrose synthesis.

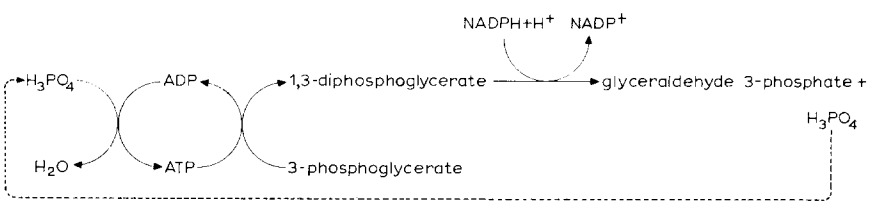
The phosphate requirement for O_2 evolution could also be met by ATP or ADP but not by AMP. A comparison of the kinetics of O_2 evolution following the addition of orthophosphate or ATP (Fig. 2) shows a much slower response to ATP, implying slow penetration, or (perhaps more probably) external hydrolysis followed by penetration of inorganic phosphate. The slowness of the response effectively ruled out clear definition of the ATP–O_2 stoichiometry since it was necessary to prolong illumination to a point at which inactivation of the chloroplasts became a major complicating factor.

The inhibition, by orthophosphate, of the O_2 evolution seen in the presence of CO_2 as the sole added substrate (Fig. 3) is perhaps best attributed to inhibition at the carboxylation step. Orthophosphate is known to inhibit ribulose-1,5-diphosphate carboxylase[12] in a competitive manner[13] and at low substrate concentrations a pronounced inhibition can be produced by a small quantity of orthophosphate[13]. In the present experiments the orthophosphate inhibition was not seen in the presence of substrate levels of ribose 5-phosphate or 3-phosphoglycerate. Large quantities of ribose 5-phosphate would act as a sink for orthophosphate producing ribulose 1,5-diphosphate in amounts which might well be able to reverse a competitive inhibition

(see ref. 13). Similarly, provision of substrate quantities of a post-carboxylation intermediate could give an O_2 evolution independent of the carboxylation step and indeed it can be demonstrated that O_2 evolution in the presence of ribose 5-phosphate is CO_2 dependent[6] whereas in the presence of added substrate concentrations of 3-phosphoglycerate there is initially no requirement for added CO_2.

We are at present at a loss to account for the partial failure of orthophosphate inhibition in the presence of excess pyrophosphate. In preliminary experiments we have confirmed the inhibition by orthophosphate of ribulosediphosphate carboxylase. Pyrophosphate does not reverse this inhibition and in fact is itself a potent inhibitor. The fact that intact isolated chloroplasts function effectively in high concentrations of pyrophosphate therefore suggests that pyrophosphate cannot continue to enter the chloroplast at a high rate (if at all) in the absence of hydrolysis. It is conceivable that excess pyrophosphate might slow the penetration of orthophosphate. Pyrophosphate is, of course, an effective complexing agent and there may be some interaction between orthophosphate, pyrophosphate and magnesium ions.

The other results reported here have a bearing on the nature of the reduction of 3-phosphoglycerate. Most workers would support the view, for which there is substantial evidence, (see *e.g.* ref. 14) that O_2 evolution in photosynthesis is associated with the transfer of hydrogen from water to ferredoxin. In order that this process might continue, the reduced ferredoxin must be reoxidised. The favoured mechanism involves the transfer of hydrogen *via* $NADP^+$ to 1,3-diphosphoglycerate (which is reduced to glyceraldehyde 3-phosphate) but there are some indications that suggest a more direct reduction of a carboxylation product[15]. The present results do not rule out this possibility but they do show quite clearly that phosphate is required for the reduction of 3-phosphoglycerate. The direct reduction of 3-phosphoglycerate to glyceraldehyde 3-phosphate is thermodynamically feasible (the free energy change is only slightly more positive than the oxidation of malate to oxaloacetate in the Krebs cycle) but the reaction catalysed by the $NADP^+$-specific triosephosphate dehydrogenase is more readily accomplished. This reaction requires the preliminary conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate (at the expense of ATP) and could account for the observed phosphate requirement. An alternative possibility is that the hydrogen donor (presumably NADPH) is formed only in a tightly coupled noncyclic photophosphorylation for which inorganic phosphate is necessary. The sequence in which 1,3-diphosphoglycerate is an intermediate requires phosphate but is not phosphate consuming.

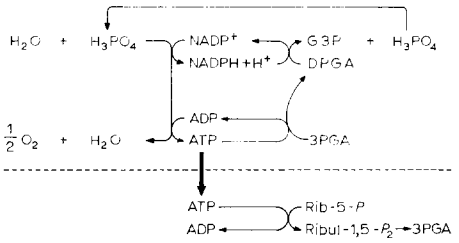


It is therefore of especial interest that while O_2 evolution in the presence of 3-phosphoglycerate was stimulated by added phosphate, the endogenous phosphate was apparently sufficient with 3-phosphoglycerate to sustain evolution for a prolonged period (Fig. 9). This contrasted with the CO_2-dependent O_2 evolution which was both

phosphate requiring and phosphate consuming (*e.g.* Fig. 1) (see also ref. 4). The consumption of phosphate in the formation of ribulose 1,5-diphosphate was strikingly underlined by the results obtained when a high concentration of pure ribose 5-phosphate was incubated with illuminated chloroplasts (Fig. 6). Clearly the most probable explanation of these results is that ribulose-5-phosphate kinase can effectively compete for available ATP so that none is available for the phosphorylation of 3-phosphoglycerate and no net O_2 evolution is seen until the addition of phosphate allows photophosphorylation to proceed at an adequate rate. Even the evolution observed in the presence of substrate concentrations of 3-phosphoglycerate was largely suppressed by the presence of ribose 5-phosphate (Fig. 8) and it is especially difficult to equate this result with a mechanism for 3-phosphoglycerate reduction which has no requirement for ATP.

Normal sequence of events (in presence of 3-phosphoglycerate) leading to $NADP^+$ regeneration, O_2 evolution and cycling of endogenous orthophosphate.

In substrate concentrations, Rib-5-*P* siphons off endogenous orthophosphate, thus suppressing $NADP^+$ regeneration and O_2 evolution.



Abbreviations: 3PGA, 3-phosphoglycerate; G3P, glyceraldehyde 3-phosphate; DPGA, 1,3-diphosphoglycerate; Rib-5-*P*, ribose 5-phosphate; Ribul-1,5-P_2, ribulose 1,5-diphosphate.

Taken together with earlier work[16] (which demonstrated the fixation of $^{14}CO_2$ and the incorporation of ^{32}P into sugar phosphates by chloroplasts illuminated in similar mixtures) the present results therefore continue to be most easily interpreted in terms of the classic Benson–Calvin carbon cycle[15]. The phosphate requirement is then consistent with the formation of 1,3-diphosphoglycerate as the substrate-level hydrogen acceptor and ribulose 1,5-diphosphate as the CO_2 acceptor.

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