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INHIBITION, BY SULPHATE, OF THE OXYGEN EVOLUTION ASSOCIATED WITH PHOTOSYNTHETIC CARBON ASSIMILATION

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SUMMARY

1. Inorganic sulphate, at $2 \cdot 10^{-2}$ M, completely inhibits the O_2 evolution associated with CO_2 fixation by entire isolated spinach chloroplasts.

2. Sulphate also inhibits O_2 production from chloroplasts provided with substrate concentrations of 3-phosphoglycerate but without added CO_2.

3. The sulphate inhibition is reversed by small quantities of orthophosphate but not by inorganic pyrophosphate. With the endogenous phosphate alone, sulphate produces a 50% inhibition at $5 \cdot 10^{-4}$ M. With $2 \cdot 10^{-4}$ M orthophosphate, $2 \cdot 10^{-3}$ M sulphate is required to produce a 50% inhibition.

4. Sulphate, at $2 \cdot 10^{-2}$ M, does not inhibit O_2 evolution from envelope-free chloroplasts utilising ferricyanide or added $NADP^+$ as the hydrogen acceptor.

5. It is concluded that sulphate does not interfere with the mechanism of O_2 evolution, or electron transport, but that by affecting photophosphorylation, or ATP utilisation, it indirectly inhibits the conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate and hence the final stages of hydrogen transfer.

INTRODUCTION

The O_2 evolution associated with CO_2 fixation by isolated chloroplasts soon ceases in the absence of added phosphate[1,2], The requirement for added phosphate can be met equally well by orthophosphate and inorganic pyrophosphate[1,2]. At higher concentrations, orthophosphate is inhibitory in reaction mixtures in which CO_2 is the sole added substrate[1–5] whereas pyrophosphate is not inhibitory, except at much higher concentrations[1–4]. During work on this problem we carried out control experiments in which sulphate was substituted for orthophosphate. At low concentrations sulphate was ineffective. It could not bring about a resumption of O_2 evolution in mixtures in which evolution had ceased for want of phosphate (*cf.* refs. 1,2). At higher concentrations sulphate was inhibitory and, like orthophosphate, it could (in some circumstances) bring about virtually complete suppression of O_2 evolution at about 10^{-2} M and appreciable changes in rate at as little as 10^{-4} M. Further work, reported below, has helped to define the nature of the sulphate inhibition.

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MATERIALS AND METHODS

Chloroplast isolation

Chloroplasts were prepared from spinach as before[2] in media containing sorbitol, 0.33 M; MES[6,7] (2-(*N*-morpholino) ethane sulphonic acid)–NaOH, pH 6.5, 0.01 M; and small quantities (1 mM) of $MnCl_2$ and $MgCl_2$ but no ascorbate or isoascorbate. They were resuspended in a similar solution except that the MES was replaced by HEPES[6,7] (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid)–NaOH (0.05 M) at pH 7.6 and EDTA (2 mM) was added.

Reaction mixtures

These contained 100 μg of chlorophyll (in a final volume of 1 ml of resuspending medium) and usually 10 μmoles of $NaHCO_3$ or 2 μmoles of 3-phosphoglycerate (sodium salt). Other additions were as indicated in the figures. When additions were made with a microsyringe during the course of an experiment the volume was kept as low as practicable (10–50 μl) and no correction made for the change in volume (1–5 %). In experiments with envelope-free chloroplasts $NaHCO_3$ and 3-phosphoglycerate were replaced by ferricyanide (10^{-3} M) or $NADP^+$ (1 μmole) *plus* ferredoxin (approx. 10 μg) prepared from parsley according to the method of HILL AND BENDALL[7] and kindly donated by Dr. R. HILL and Dr. JANET WEST.

Envelope-free chloroplasts

These were prepared as before[2] from entire chloroplasts simply by adding a chloroplast suspension to sufficient water to decrease the molarity of sorbitol, briefly, to less than 0.04 M. Resuspending medium at double the usual concentration was then added so that the final concentration and volume of the reaction mixtures was as above.

O_2 measurement

O_2 was measured polarographically (*cf.* refs. 9,10) using a twin-pen recorder (Rikadenki, Model B241) and two identical perspex cells in which basal Clark-type electrodes (platinum–KCl–silver) were separated from magnetically stirred reaction mixtures by a teflon membrane. The cells were fitted with perspex jackets through which water at 20° was passed at about 5 l/min. Approx. 3 min temperature equilibration was allowed prior to illumination. A value of 0.28 μmole of O_2 was taken as the difference between the electrical output of the cells when these contained 1 ml of air-saturated water at 20° and the equilibrium value after the addition of sodium dithionite. This agreed with values recorded following step-wise addition of standardized H_2O_2 to catalase dissolved in resuspending medium.

Illumination

Each cell was illuminated by a quartz-iodine (150 W, 24 V) slide projector. The light beam was passed through 14 cm of water, a Balzer heat filter (Calflex c, Tempax) and a red perspex filter, transmitting approx. 80 % of the incident light at wavelengths higher than 600 mμ.

RESULTS

Inhibition, by sulphate, of O_2 evolution with CO_2 as sole substrate

Fig 1. shows the course of O_2 evolution from illuminated spinach chloroplasts in mixtures containing CO_2 as the sole added substrate and in which the phosphate requirement was met by a small quantity (10^{-3} M) of inorganic pyrophosphate[1-4]. Addition of sulphate (as indicated) to a final concentration of 10^{-2} M caused a rapid and progressive inhibition. When net evolution had ceased, the addition of orthophosphate (to 10^{-3} M) initiated a slight but progressive reversal of inhibition. Addition of orthophosphate to the control was without effect. Comparable inhibitions have been demonstrated in mixtures containing CO_2 together with substrate concentrations of ribose 5-phosphate or dihydroxyacetone phosphate. The reversals by orthophosphate were then much more marked so that the results resembled those obtained with 3-phosphoglycerate (see Fig. 2 and below). Inorganic pyrophosphate did not bring about reversal, or protect against inhibition.

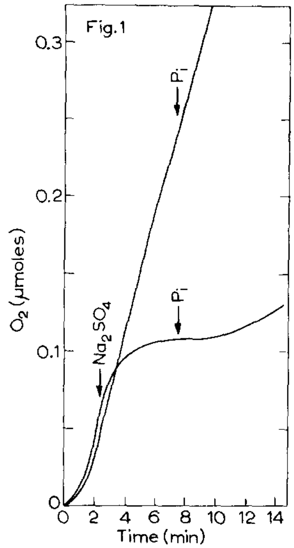


Fig. 1. O_2 evolution by chloroplasts during photosynthetic carbon assimilation and its inhibition by sulphate. Both reaction mixtures contained chloroplasts and bicarbonate (see MATERIALS AND METHODS) and pyrophosphate (1 μmole). The addition of sulphate (10 μmoles) to one produced a progressive inhibition. When net evolution had ceased the further addition of orthophosphate (1 μmole) induced renewed evolution.

Inhibition, by sulphate, of O_2 evolution with 3-phosphoglycerate as sole substrate

There are grounds for believing that intact chloroplasts are capable of catalysing the entire photosynthetic carbon cycle (see *e.g.* refs. 11–16) and it has been demonstrated that O_2 evolution in systems such as those referred to in the preceding section soon ceases in the absence of added bicarbonate and restarts following its addition[9,16]. Initially, however, the O_2 evolution seen in the presence of substrate quantities of 3-phosphoglycerate is largely independent of carboxylation[16] even if CO_2 is present[3]. Fig. 2 shows that oxygen evolution by chloroplasts in mixtures containing 3-phosphoglycerate (but no added bicarbonate) was also suppressed by sulphate and that the inhibition was reversed by orthophosphate. The degree of reversal was more marked than it was in mixtures containing CO_2 alone (*cf.* Fig. 1) presumably because orthophosphate (at this concentration) itself inhibits when CO_2 is the sole substrate whereas it stimulates if 3-phosphoglycerate is present (see ref. 2). For this reason it was difficult to design single experiments which would provide unequivocal evidence of reversal. Thus if optimal concentrations of orthophosphate were used initially, the degree of sulphate inhibition was small. Conversely, if the orthophosphate concentration was initially sub-optimal a further addition of orthophosphate would bring about an acceleration whether sulphate was present or not. However, it will be seen that in Fig. 2 the conditions were selected to give a considerable inhibition by sulphate and that subsequent addition of orthophosphate increased the rate by a factor of 6 whereas the same addition to the control (no sulphate) increased the rate by a factor of only 1.4 (see also Fig. 4). Fig. 3, otherwise similar to Fig. 2, shows that this reversal could not be brought about by inorganic pyrophosphate (which was also ineffective when added in several smaller or larger quantities).

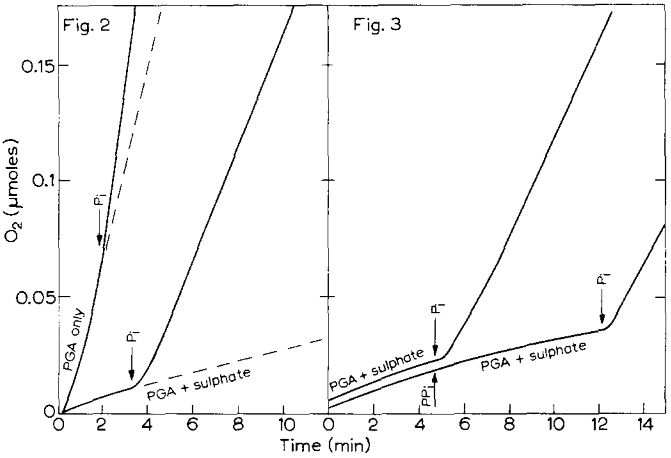
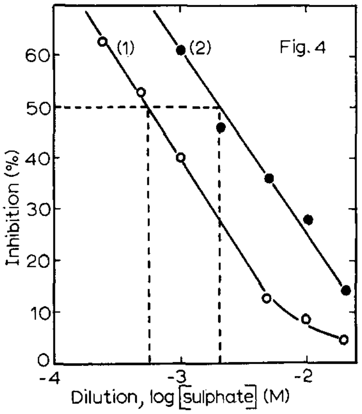


Fig. 2. Inhibition, by sulphate, of O_2 evolution with 3-phosphoglycerate (PGA) as substrate and its reversal by orthophosphate. Both reaction mixtures contained chloroplasts, pyrophosphate (1 µmole) and 3-phosphoglycerate (2 µmoles) and one contained enough sulphate (20 µmoles) to cause a marked inhibition. Subsequent addition of orthophosphate (1 µmole, as indicated) largely reversed the sulphate inhibition.

Fig. 3. Conditions initially as for Fig. 2 but with 10^{-2} M sulphate in both vessels. The additions of orthophosphate (1 µmole, as indicated) brought about reversal (*cf.* Fig. 2) but the simultaneous addition of pyrophosphate (5 µmoles) to the second vessel was without effect. The subsequent addition of orthophosphate (1 µmole to the second vessel, as indicated) brought about reversal as before. PGA, 3-phosphoglycerate.

Concentration of sulphate required to bring about 50 % inhibition

Since O_2 evolution by isolated chloroplast has a phosphate requirement[1,2] and since sulphate inhibition is reversed by orthophosphate, these two parameters can not be considered independently. Fig. 4 shows the degree of inhibition brought about by several concentrations of sulphate in the presence of added orthophosphate. It compares this with the corresponding effects observed in the presence of endogenous phosphate alone. Reversal of inhibition (or protection against inhibition), by orthophosphate, is implicit in these data. It will be seen that in the absence of added phosphate, $5 \cdot 10^{-4}$ M sulphate was sufficient to produce a 50% inhibition. In the presence of added orthophosphate ($2 \cdot 10^{-4}$ M) it was necessary to increase the sulphate to $2 \cdot 10^{-4}$ M in order to produce a 50% inhibition.



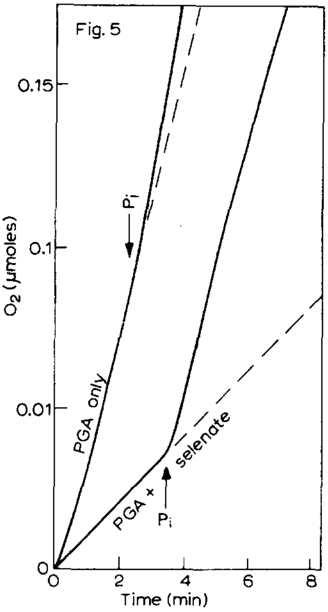


Fig. 4. The extent of sulphate inhibition in the presence of endogenous, and added, orthophosphate. O_2 evolution was measured (as in Figs. 2 and 3) in the presence of 3-phosphoglycerate (2 μmoles) and various concentrations of sulphate (as indicated) and the initial rates expressed (Curve 1) as a percentage of a control containing no sulphate. This was repeated (Curve 2) using mixtures containing added orthophosphate (0.2 μmole). Similar results were obtained when selenate was substituted for sulphate (see text).

Fig. 5. Inhibition, by selenate, of O_2 evolution with 3-phosphoglycerate (PGA) as substrate and its reversal by orthophosphate. As for Fig. 2 but with selenate (10 μmoles) instead of sulphate.

Inhibition by selenate

Selenate was also inhibitory, but less so than sulphate. The selenate inhibition was also reversed by orthophosphate (Fig. 5). An experiment of the type illustrated in Fig. 4 (but with selenate instead of sulphate) showed that in the presence of endogenous phosphate, approx. $4 \cdot 10^{-3}$ M selenate was required to bring about a 50% inhibition. In the presence of $2 \cdot 10^{-4}$ M orthophosphate (in addition to the endogenous phosphate) approx. $2 \cdot 10^{-2}$ M selenate was required to bring about a comparable degree of inhibition.

Effect of sulphate on electron transport

Chloroplasts prepared in sugar media retain their envelopes to a considerable extent[15]. If briefly exposed to media of low osmotic pressure they lose their envelopes and virtually all of their ability to fix CO_2[17] (see also ref. 18) or to evolve O_2 in mixtures in which 3-phosphoglycerate or CO_2 serve as precursors of the hydrogen acceptor[2].

However, their ability to evolve O_2 in mixtures containing substrate concentrations of ferricyamide or $NADP^+$ is enhanced. For these reasons, experiments in which ferricyanide or exogenous $NADP^+$ were used as the hydrogen acceptor were carried out with envelope-free chloroplasts. Figs. 6 and 7 show that sulphate was without detectable effect in such systems when used at a concentration ($2 \cdot 10^{-2}$ M) which was sufficient to eliminate the oxygen evolution associated with carbon assimilation (*cf.* Fig. 1). In Fig. 6, one trace records O_2 evolution from a reaction mixture containing $2 \cdot 10^{-2}$ M sulphate and osmotically shocked chloroplasts *plus* ferricyanide while the second trace is a record of evolution from an otherwise identical control initially without sulphate. It will be seen that the rates were identical both before and after the addition of a small quantity of sulphate (10^{-4} M) to the control. The subsequent addition of NH_4Cl (as an uncoupling agent) brought about marked increases in rates. The uncoupled O_2 evolution (Fig. 6) was also entirely unaffected by the concentration of sulphate. Fig. 7 illustrates a similar experiment in which the ferricyanide was replaced by substrate concentrations of $NADP^+$ together with purified ferredoxin. Again it will be seen that sulphate was without detectable effect prior to, or after, uncoupling by NH_4Cl.

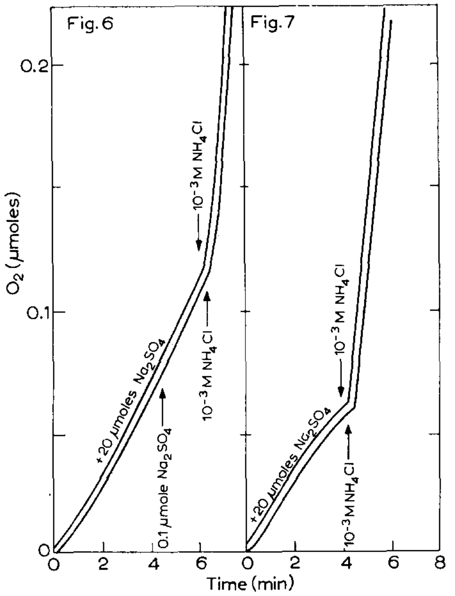


Fig. 6. Failure of sulphate to affect O_2 evolution by envelope-free chloroplasts with ferricyanide as the oxidant. Both mixtures contained envelope-free chloroplasts (see MATERIALS AND METHODS) and ferricyanide (10^{-3} M). One also contained sulphate (20 μmoles) and sulphate (0.1 μmole) was added to the second as indicated. NH_4Cl was subsequently added to both, as indicated, to a final concentration of 10^{-3} M.

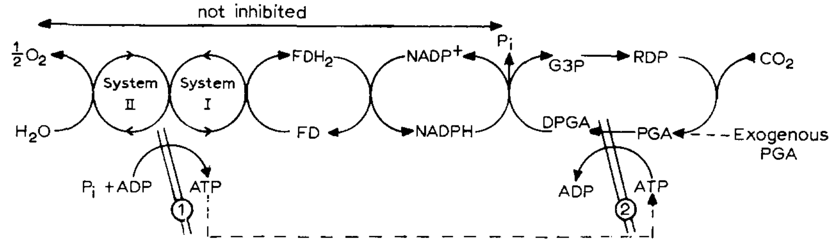
Fig. 7. Failure of sulphate to affect O_2 evolution by envelope-free chloroplasts with $NADP^+$ (in substrate concentration) as the hydrogen acceptor. As in Fig. 6 but with $NADP^+$ and ferredoxin (see MATERIALS AND METHODS) in place of ferricyanide. One mixture contained sulphate (20 μmoles), the other was without. NH_4Cl was subsequently added to both to a final concentration of 10^{-3} M.

DISCUSSION

The results show that sulphate inhibits the O_2 evolution associated with CO_2 assimilation by entire chloroplasts (Fig. 1). It also inhibits the O_2 production (which is initially independent of added CO_2[3,16]) in similar mixtures containing substrate concentrations of 3-phosphoglycerate. Conversely, it does not inhibit the O_2 evolution mediated by envelope-free chloroplasts in which ferricyanide (or NADP$^+$) serves as the hydrogen acceptor (Scheme I). Accepting present views (for a recent review see ref. 14) this would appear to leave only two susceptible reactions:

3-Phosphoglycerate + ATP → 1,3-diphosphoglycerate + ADP (1)

1,3-Diphosphoglycetate + NADPH → glyceraldehyde 3-phosphate + orthophosphate + NADP$^+$ (2)



Scheme I. Sulphate probably competes with phosphate at Site 1 therefore inhibiting formation of hydrogen acceptor at Site 2. Electron transport from water to NADP$^+$ (and associated O_2 evolution) is not affected. FD, ferredoxin; G3P, glyceraldehyde 3-phosphate; RDP, ribulose 1,5-diphosphate; PGA, 3-phosphoglycerate; DPGA, 1,3-diphosphoglycerate.

Because the sulphate inhibition is so readily reversed by orthophosphate it is difficult to resist the notion that the first of these reactions is the one affected and that the inhibition is indirect, resulting from competition between sulphate and phosphate in photosynthetic phosphorylation. This could be simple competition for active sites on an enzyme or it might, in turn, derive from interference with the movement of solutes across chloroplast membranes. In searching for mechanisms involving ATP, it must also be borne in mind that plant enzymes can catalyse the formation of adenosine 5′-phosphosulphate and 3′-phosphoadenosine 5′-phosphosulphate[19,20]. Since these enzymes will also catalyse the formation of adenosine selenates[20], it is of interest that selenate behaves like sulphate (Fig. 4) but at a higher concentration.

Inhibition of ATP formation or the provision of alternative means of ATP utilisation, would also interfere with CO_2 assimilation (Fig. 1) by slowing the conversion of ribulose 5-phosphate to ribulose 1,5-diphosphate. In addition, sulphate is a known inhibitor of ribulose diphosphate carboxylase[21]. However, neither reaction would be initially involved in the simpler system (Figs. 2–4) in which 3-phosphoglycerate is provided as a substrate.

To ourselves, one of the most interesting aspects was the failure of inorganic pyrophosphate to bring about reversal of sulphate inhibition. In meeting the phosphate requirement for photosynthesis, one molecule of pyrophosphate acts like two molecules of orthophosphate, yet pyrophosphate is not inhibitory until the concentration is increased by a factor of 100[1,2]. Thus although pyrophosphate can substitute for orthophosphate in some respects it does not in others. The present results therefore reinforce our previous view that the passage of pyrophosphate through the chloroplast envelope (and any prior or subsequent hydrolysis) occurs only in a controlled manner.

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