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# INORGANIC SULFATE AND SELENATE AS ENERGY TRANSFER INHIBITORS OF PHOTOPHOSPHORYLATION

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## SUMMARY

1. Inorganic sulfate or selenate inhibits photophosphorylation in lettuce chloroplast fragments. The inhibition was found to be competitive with inorganic phosphate in all systems tested (photophosphorylation, post-illumination ATP formation, acid–base phosphorylation) with a $K_i$ for sulfate of around 2 mM.

2. Sulfate or selenate inhibited only the fraction of electron transport stimulated by coupled phosphorylation. The basal electron transport rate was nearly unaffected. Sulfate inhibited also the arsenate-stimulated electron transport rate, but not the stimulation caused by other uncouplers.

3. Sulfate and selenate inhibit light-triggered ATP–$P_i$ exchange, but not the light-triggered Mg-ATPase. The concentrations needed for 50% inhibition are higher than those needed to inhibit phosphorylation.

4. The results indicate that sulfate or selenate act as energy transfer inhibitors of photophosphorylation, competing with $P_i$ at its binding site.

## INTRODUCTION

One of the approaches in investigations of the mechanism of photophosphorylation has been through the use of phosphate analogues. Arsenate[1] and thiophosphate[2] have been shown to compete with inorganic phosphate. Inorganic sulfate and selenate were also shown to inhibit photophosphorylation[3]. However, there was not complete agreement concerning their mechanism of inhibition[3–6].

The inhibition by sulfate was found to be competitive with inorganic phosphate both in photophosphorylation[4] and in $O_2$ evolution coupled to $CO_2$ or phosphoglycerate reduction[5]. The rate of ferricyanide or NADP reduction was found to be accelerated[4] or unaffected[3,5] by sulfate. Sulfate, in the presence of $Mg^{2+}$ and ADP under coupled electron flow conditions was reported to cause an irreversible inactivation of the coupling factor[6]. This effect was found to be non-competitive with respect to $P_i$ or ADP[6]. It was also reported that the light-dependent formation of coupling factor–ADP complex from AMP and $P_i$, is inhibited by either arsenate or sulfate[7].

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Abbreviations: FCCP, Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, Dicyclohexyl carbodiimide.

Our present findings indicate that inorganic sulfate acts as an energy transfer inhibitor. It seems that its site of interaction is identical to the binding site for $P_i$.

## METHODS

### *Chloroplast preparation*

50 g of lettuce leaves were cut into small pieces and placed inside a blendor jar with 125 ml of an ice cold solution of 0.2 M sucrose –0.1 M NaCl–0.02 M sodium ascorbate– 50 mM Tris, pH 7.8, and a very small amount of antifoam A. After blending 5 s at line voltage and 25 s at 85% of line voltage, the suspension was filtered through four layers of gauze and centrifuged at 500×*g* for 90 s (pellet discarded). The supernatant was centrifuged at 1000×*g* for 7 min. The pellet was resuspended in 40 ml 0.2 M sucrose–0.1 M KCl solution, centrifuged at 500×*g* for 90 s (pellet discarded) and the supernatant centrifuged at 1500×*g* for 10 min. This final pellet was resuspended in sucrose–KCl solution (minimal volume). The chlorophyll concentration obtained was 2–4 mg chlorophyll/ml.

For acid–base transition experiments the chloroplasts were resuspended in 10 mM NaCl at 4 °C for 20 min, at a final concentration of 0.1 mg chlorophyll/ml sedimented at 10000×*g* for 10 min, and resuspended in 10 mM NaCl to a concentration of about 5 mg chlorophyll/ml.

Diquat reduction[8] ferrocyanide formation[9,10], ATP formation[11] "Xe" experiments[12], acid–base experiments[13], the determination of the thylakoid osmotic space[14] and the evaluation of $\Delta$pH from the quenching of the fluorescence of 9-amino acridine[15], were performed as previously described.

The activation of $Mg^{2+}$-ATPase and ATP–$P_i$ exchange was performed in a special glass holder with two side arms[16].

## RESULTS AND DISCUSSION

Fig. 1 illustrates the inhibition of ferricyanide photoreduction and the photophosphorylation coupled to it by sulfate. The rate of ferricyanide reduction in the absence of phosphorylation (without $P_i$) is seen to be only slightly affected by the presence of sulfate. Similar results were obtained when diquat replaced ferricyanide as the electron acceptor. A kinetic analysis was made of the inhibition of photophosphorylation by $SO_4^{2-}$ at two pH values: 6.5 and 8.5. Typical competition curves between sulfate and $P_i$ were found with similar $K_i$ values for sulfate (1–2 mM).

Fig. 2 shows that the inhibition of photophosphorylation by sulfate was roughly equally efficient between pH 6.0 and 9.0. Changing the ionic strength of the medium (15–90 mM KCl) did not affect the efficiency of photophosphorylation inhibition by sulfate (not shown). The inhibition by sulfate was constant with reaction times up to 5 min.

Fig. 3 demonstrates the inhibition of the rate of electron transport by sulfate at different external pH values. It is evident that only the phosphorylation-coupled electron transport is inhibited whereas the basal electron transport is essentially unaffected. The maximal inhibition by sulfate appears at pH 7.5, where also the stimulation of electron transport by $P_i$ is maximal. Arsenate-stimulated electron transport was also competitively inhibited by sulfate (Table I). However, the

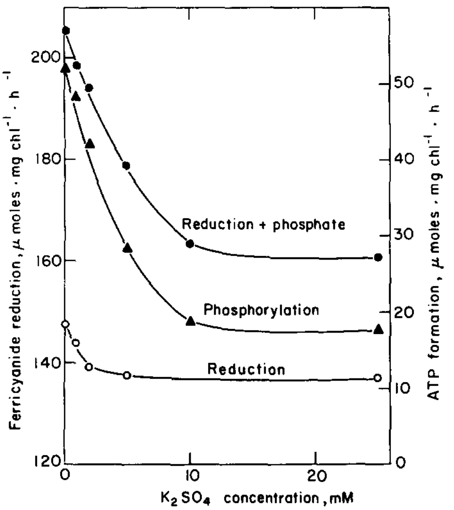


Fig. 1. Inhibition of ferricyanide photoreduction and phosphorylation by sulfate. The reaction mixture contained, in a total volume of 3 ml: 30 mM Tricine–NaOH, pH 7.5; 4 mM $MgCl_2$; 0.3 mM $K_3Fe(CN)_6$; 1.33 mM ADP, pH 7.5; and chloroplasts containing 32 μg chlorophyll. Where indicated inorganic phosphate 1 mM (containing $5 \cdot 10^7$ cpm $^{32}P$) and sulfate at the indicated concentrations were added. KCl was added at 75 mM to the sulfate-less samples, and at an appropriate lower concentration to the rest in order to keep all systems at the same osmolarity. The reaction was run at 20 °C for 2 min in saturating white light, and terminated by the addition of trichloroacetic acid to a final concentration of 3%. Following centrifugation-samples of the supernatant were taken to determine ATP and ferrocyanide formed[9] as described under Methods. chl, chlorophyll.

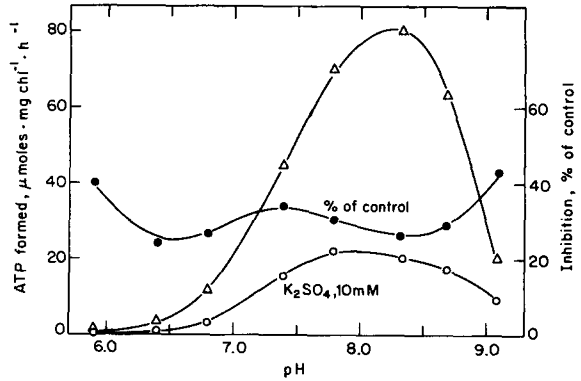


Fig. 2. Inhibition of photophosphorylation by sulfate at different external pH values. The reaction mixture was the same as that described under Fig. 1, except for the buffers used (30 mM Tricine–maleate–NaOH, for pH 5.9–7.8; and Tricine–glycine–NaOH for pH 8.35–9.1), the inorganic phosphate concentration which was 0.5 mM and the KCl concentration which was 15 mM in the sulfate-less samples. chl, chlorophyll.

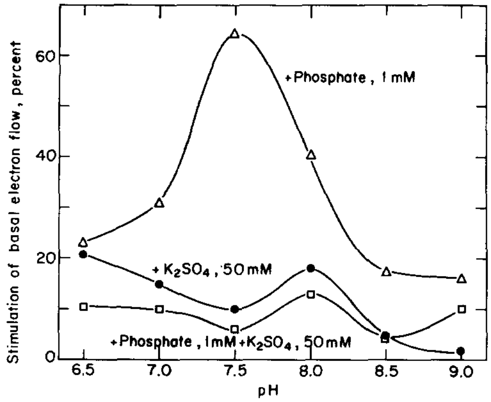
stimulation by the other uncouplers tested was unaffected by sulfate. This is consistent with the notion that the site of interaction of $NH_4Cl$, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and atebrin precedes that of sulfate (and arsenate). Selenate behaved similar to sulfate, as an inhibitor (Table 1).

TABLE I

EFFECT OF SULFATE AND SELENATE ON ELECTRON TRANSPORT

The reaction mixture contained, in a total volume of 3.0 ml: 30 mM Tricine–NaOH, pH 7.5, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM $K_3Fe(CN)_6$, 0.4 mM ADP, 20 μM ATP, and chloroplasts containing 20 μg of chlorophyll. Ferricyanide reduction was followed spectroscopically as previously described[10]. 0 control refers to 113 μmoles ferricyanide reduced per mg chlorophyll per h.

| *Additions* | *Ferricyanide reduction rate (percent stimulation)* | | |
|---|---|---|---|
| | *Control* | *Plus 25 mM $K_2SO_4$* | *Plus 25 mM $Na_2SeO_4$* |
| None | 0 | 12 | 15 |
| + Arsenate, 1.25 mM | 115 | 10 | 30 |
| + Arsenate, 2.5 mM | 132 | — | 54 |
| + Arsenate, 25 mM | 270 | 240 | 270 |
| + $NH_4Cl$, 3.3 mM | 305 | — | 280 |
| + FCCP, 1 μM | 300 | 280 | 300 |
| + Dianemycin, 5 μM | 515 | — | 515 |



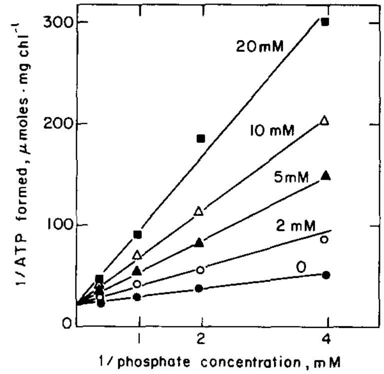


Fig. 3. Dependence of the inhibition of ferricyanide reduction by sulfate on pH. The reaction mixture was the same as that described under Fig. 1, except for the buffers which were 30 mM Tricine–maleate–NaOH, for pH values 6.5–7.5 and Tricine–glycine–NaOH for pH values 8.0–9.0. The ordinate indicates stimulation of the rate of reduction relative to a control (*i.e.* basal electron flow, no phosphate) containing 75 mM KCl, by the additions indicated in the figure (where 50 mM $K_2SO_4$ was added, the 75 mM KCl was omitted).

Fig. 4. Kinetic analysis of the competition between sulfate and phosphate in the dark stage of post-illumination ATP formation. The light stage reaction mixture contained in a total volume of 1.5 ml in μmoles: Tricine-maleate, pH 6.0, 2.0; PMS, 0.05; and chloroplasts-containing 200–250 μg of chlorophyll. This mixture was illuminated in a syringe at 20 °C for 30 s and immediately transferred to the dark reaction mixture at 0 °C containing, in a total volume of 0.75 ml, in μmoles: Tricine-glycine, pH 8.5, 6.0; $MgCl_2$, 8; ADP, pH 8.5, 0.5; inorganic phosphate, pH 8.5 (containing $10^7$ cpm $^{32}P$), 1.25; sulfate at the indicated concentrations and KCl at the concentration needed to keep all mixtures iso-osmolar (30 mM in the sulfate-less mixtures). The reaction was terminated after 15 s incubation in the dark by the addition of trichloroacetic acid to a final concentration of 3%, $K_2SO_4$ concentrations are indicated on the figure. chl, chlorophyll.

TABLE II

EFFECT OF SULFATE ON POST-ILLUMINATION ATP FORMATION

The light reaction mixture contained, in a total volume of 1.0 ml: 5 mM Tricine–maleate, pH 6.0 or 8.0, 45 mM NaCl, 60 μM PMS, and chloroplasts containing 450 μg of chlorophyll. The dark reaction contained: 30 mM Tricine– NaOH, pH 8.5, 45 mM NaCl, 2 mM ADP, 5 mM $MgCl_2$, and phosphate (containing $5 \cdot 10^8$ cpm $^{32}P$), as indicated. 100% refers to 43 and 74 nmoles ATP formed per mg chlorophhyll in the upper and lower experiments, respectively.

| *Light phase* | | *Dilution between phases* | *Additional compounds in dark phase* | *ATP formed (%)* |
|---|---|---|---|---|
| *Additional Compounds* | *pH* | | | |
| None | 6.0 | 1:20 | phosphate, 1mM | (100) |
| $K_2SO_4$, 10 mM | 6.0 | 1:20 | phosphate, 1 mM | 99 |
| None | 6.0 | 1:20 | phosphate, 1mM *plus* $K_2SO_4$, 10mM | 38 |
| None | 6.0 | 1:2 | phosphate, 0.25 mM | (100) |
| $K_2SO_4$, 5 mM | 6.0 | 1:2 | phosphate, 0.25 mM | 34 |
| None | 6.0 | 1:2 | phosphate, 0.25 mM *plus* $K_2SO_4$,2.5 mM | 35 |
| None | 8.0 | 1:2 | phosphate, 0.25 mM | 7 |
| $K_2SO_4$, 5 mM | 8.0 | 1:2 | phosphate, 0.25 mM | 3 |
| None | 8.0 | 1:2 | phosphate, 0.25 mM plus $K_2SO_4$, 2.5 mM | 3 |

The effect of sulfate on post-illumination photophosphorylation ("Xe" system) was tested by adding it either before the light stage or only to the dark stage. Equal inhibitions were obtained indicating that sulfate affected mostly the dark stage. A kinetic analysis of the interaction between sulfate and phosphate during the dark stage is shown in Fig. 4. The competitive nature of the inhibition with $P_i$ is again clearly evident, with a $K_i$ for sulfate of 2 mM.

To investigate the possible effect of sulfate on the light phase, two approaches were taken: In the first, the light stage medium underwent 1:20 dilution on passing to the dark stage[12]. In this way the concentration of $SO_4^{2-}$ was 10 mM in light stage, but was diluted to an ineffective concentration of 0.5 mM during the dark stage. Table II demonstrates the lack of inhibition by sulfate during the light stage. In the second, we made use of the competition between sulfate and phosphate in the dark stage and raised the phosphate concentration in the dark phase to a point where sulfate was no longer inhibitory. Here, too, no inhibition by sulfate during the light stage was observed (not shown). This was true also when the light stage was conducted at pH 8.0 (Table II). A similar experiment conducted in the presence of phenylenediamine, which considerably increases phosphorylation even at light stage pH of 8.0 (ref. 24), yielded similar results.

Sulfate was also found to be a potent inhibitor of the acid–base phosphorylation system. When included only in the "basic -phase", sulfate again competed with $P_i$ with a $K_i$ of 3 mM. Sulfate also inhibited the acid phase, which may be due to the non-specific effect on the coupling factor exerted also by other anions proposed earlier[18].

It was reported recently that phosphorylation conditions lower the steady state $\Delta$pH formed across the thylakoid membrane in the light[19–22]. We observed[22] that ADP+$P_i$ or ADP+arsenate lower the steady state $\Delta$pH, whereas Dio-9 reverses this effect. In a similar manner, selenate added in excess over arsenate or phosphate partially restored the high $\Delta$pH[22].

It was previously reported[23] that the energy transfer inhibitors Dio-9 and phloridzin inhibit the dithiothreitol activated Mg-ATPase and ATP–$P_i$ exchange reaction. Since selenate or sulfate were shown above to act as energy transfer inhibitors which act competitively with phosphate, they would be expected to behave like arsenate[24], inhibit the ATP–$P_i$ exchange reaction but not the Mg-ATPase. Table III shows that this indeed was found to be the case. Half-maximal inhibition was obtained at selenate/$P_i$ of about 20 and sulfate/$P_i$ of about 30. These ratios are higher than those needed to inhibit photophosphorylation in the various systems. This may be due to a change in affinity for the $P_i$-binding site, due to the light-triggering conditions as was previously observed for ADP[25].

The data presented here seem to indicate that sulfate and selenate act as energy transfer inhibitors of photophosphorylation in chloroplasts. The inability of other workers to observe inhibition of electron transport by sulfate may be due to several reasons: (a) At high pH values the typical energy transfer inhibitors Dio-9, dicyclohexyl carbodiimide (DCCD) and phlorizin inhibit the reduction of ferricyanide under phosphorylation conditions below its basal value (without ADP)[26]. In contrast, sulfate did not depress the rate of coupled electron flow below the basal rate, even at these high pH values (Fig. 3). The effect is therefore relatively small and strongly dependent on the external pH. (b) To observe significant inhibition, a high ratio of sulfate/$P_i$ is needed since the $K_i$ for sulfate is about five times higher than the $K_m$ for $P_i$. However, at low $P_i$ concentrations the stimulation of the basal rate of electron flow by the phosphorylation reagents is small, and the inhibition is therefore hard to follow. On the other hand, very high sulfate concentrations result in partial uncoupling.

TABLE III

SULFATE AND SELENATE INHIBITION OF LIGHT-TRIGGERED REACTIONS

The central arm[16] contained the triggering medium: 20 mM Tricine–NaOH, pH 8.0, 20 mM KCl; 5 mM $MgCl_2$, 0.5 mM inorganic phosphate (containing $2 \cdot 10^6$ cpm $^{32}P$), $10^{-5}$ M PMS, 10 mM dithiothreitol, and chloroplasts containing 30–40 μg chlorophyll in 0.8 ml. After 5 min illumination with saturating light at 20 °C the light was turned off and the content of the side-arm (5 μmoles ATP *plus* the other additions indicated in 0.2 ml) were mixed with the central arm. The reaction taking place in the dark was terminated after 10 min by the addition of trichloroacetic acid to a final concentration of 3%. After centrifugation 0.5 ml were taken to determine $^{32}P$ incorporation into ATP[10], and 0.2 ml to determine $P_i$ released[27]. 100% ATP–$P_i$ exchange was 32 μmoles $^{32}P_i$ incorporated into ATP per mg chlorophyll per h. 100% ATPase was 46 μmoles $P_i$ released per mg chlorophyll per h.

| *Salt added to dark phase* | *Concentration (mM)* | *ATP–$^{32}P$ exchange (% control)* | *Mg-ATPase (% control)* |
|---|---|---|---|
| KCl | 75 | (100) | (100) |
| $K_2SO_4$ | 50 | 47 | 106 |
| $Na_2SeO_4$ | 50 | 27.4 | 95 |

A valuable difference from other energy transfer inhibitors may be the selective inhibition of light-triggered ATP–$P_i$ exchange with no effect on the light-triggered Mg-ATPase.

It is of interest to contrast the effect of arsenate with that of sulfate or selenate. Both compete with $P_i$ and with each other, indicating that they interact with the same site (see also ref. 7). However, whereas arsenate acts as an uncoupler, sulfate and selenate act as energy transfer inhibitors. They may all bind to the $P_i$ binding site, but unlike arsenate, sulfate or selenate may be unable to interact with ADP and thus will not lead to the dissipation of the high energy state and prevent its unloading by $P_i$ or arsenate.

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