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THE INTERACTION OF NITRITE WITH PHOTOSYNTHETIC ELECTRON TRANSPORT UNDER ANAEROBIC CONDITIONS

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Summary

Chlorella cells were examined in a modulated oxygen polarograph under aerobic and anaerobic conditions. At light intensities below about 600 ergs · $cm^{-2} \cdot s^{-1}$ of 650 nm light, the oxygen yield and phase lag are lower under anaerobic conditions. Addition of 25 mM sodium nitrite increases both these parameters to values close to those found in the presence of oxygen. It is proposed that nitrite is reduced by Photosystem I thus diverting electrons from the cyclic electron transport pathway. The intersystem electron transport chain becomes more oxidized and this suppresses a backflow of electrons to the oxidizing side of Photosystem II, hence increasing the oxygen yield and the phase lag. The removal of oxygen from the bathing medium also alters the response of dark adapted *Chlorella* to a series of saturating light flashes. In terms of the Kok model of Photosystem II (Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457—475) there is a large increase in the parameter α. Addition of nitrite reverses this change and virtually restores the response seen in the presence of oxygen. The deactivation of the S_2 state is greatly speeded up in the absence of oxygen but the addition of nitrite again reverses this.

Introduction

The presence of oxygen can influence photosynthesis in a number of ways. For example it can react with ribulose bisphosphate in a reaction catalyzed by ribulosebisphosphate carboxylase (EC 4.1.1.39). This process is called photorespiration and causes a depression of the rate of carbon dioxide fixation compared with the rate observed in the absence of oxygen. Oxygen can also act as

an electron acceptor to Photosystem I in isolated chloroplasts, the well known Mehler reaction [1]. A third site of interaction was proposed by Diner and Mauzerall [2] who suggested that oxygen could accept electrons from reduced plastoquinone in *Chlorella*. They proposed this role for oxygen to explain the depression in photosynthetic oxygen yield which they observed at low light intensities under anaerobic conditions. Their proposal was disputed by Schreiber and Vidaver [3] who studied fluorescence emission from *Scenedesmus* and who suggested that oxygen could alter the redox state of plastoquinone only indirectly. According to Schreiber and Vidaver, oxygen could accept electrons from Photosystem I and by so doing, could alter the rate at which electrons could return via a cyclic pathway to plastoquinone. In a recent paper from this laboratory (Sinclair et al. [4]) it was shown that the phase lag and the oxygen yield of the oxygen signal from *Chlorella* observed in a modulated oxygen electrode were lowered by the removal of oxygen at low light intensities. A model was presented to explain these results in which electrons could flow from an electron transport pool between the two photosystems to one on the oxidizing side of Photosystem II thus suppressing the oxygen yield and decreasing the phase lag. Oxygen could suppress this movement of electrons by oxidizing the intersystem pool either directly as Diner and Mauzerall [2] suggested or indirectly as Schreiber and Vidaver [3] proposed.

In this paper we report on experiments intended to investigate these phenomena more fully. *Chlorella* cells were exposed to nitrite, nitrate and glucose to see if any of these substances could alter the decreases in oxygen yield and phase lag observed previously.

Methods

The organism used in this study was *Chlorella vulgaris* obtained from the Carolina Biological Supply Co. and cultivated as described previously (Sinclair and Arnason [5]). Monolayers of *Chlorella* cells were deposited on the shiny platinum electrode in a modulated oxygen electrode [5] and both the relative rate of oxygen evolution and the phase lag between the oxygen signal and the light modulations were observed. The modulation frequency used in these experiments was 16 Hz and the modulated light beam was passed through a 650 nm interference filter before reaching the cells. The light intensity was varied by means of neutral density filters. The bathing medium contained 100 mM sodium chloride and 10 mM Tris (hydroxymethyl)aminomethane hydrochloride (Tris), pH 7.6. This medium was equilibrated with a gas mixture containing 95% air and 5% carbon dioxide to produce an aerobic environment for the cells but was equilibrated with 95% nitrogen and 5% carbon dioxide to produce anaerobic conditions. Sodium nitrite, sodium nitrate and glucose were added to this medium as indicated below.

Dark adapted *Chlorella* were also studied in an oxygen electrode by subjecting them to a series of saturating light flashes using an apparatus described in Arnason and Sinclair [6]. The pattern of oxygen pulses produced in response to the light flashes was observed under aerobic and anaerobic conditions and also in an anaerobic solution to which 1 mM sodium nitrite had been added. The deactivation of the S_2 state of Kok's model of Photosystem II [7] was

observed under these same three sets of conditions. Dark adapted *Chlorella* were subjected to a single flash followed by a dark period of length Δt seconds and then illuminated with two flashes separated by 0.3 s. The yield of the third flash was taken to be proportional to the number of charge accumulation centres still in the S_2 state after time Δt. All experiments were performed at 21°C.

Results

The relative rates of oxygen revolution and the corresponding phase lags obtained over a range of light intensities for three different conditions are shown in Fig. 1. Under aerobic conditions the rate of oxygen evolution (large squares) varies linearly with light intensities while the phase lag (small squares) is invariant. For anaerobic conditions in the absence of sodium nitrite, the rate of O_2 evolution (large circles) is lower than the value for the aerobic situation at low light intensities but higher at high light intensities. The phase lag (small circles) is smaller under anaerobic conditions and also decreases with light intensity. These results are very similar to those reported earlier [4]. For anaerobic conditions in the presence of 25 mM sodium nitrite, the rate of oxygen evolution (large triangles) at low light intensities is close to the aerobic values whereas at high intensities it is almost identical to the value observed under anaerobic conditions in the absence of nitrite. The phase lag in the presence of nitrite and the absence of oxygen (small triangles) does not vary with light intensity and is equal to that observed under anaerobic conditions in the absence of nitrite at high light intensities. The addition of 25 mM sodium nitrite in the presence of oxygen or of 25 mM sodium nitrate in the presence or absence of oxygen had no observable effects. When 10 mM glucose was added to an anaerobic solution and the mean light intensity was 900 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}, the phase lag decreased by 5° and the relative rate of oxygen evolution fell by 20%.

Chlorella cells were exposed to different concentrations of sodium nitrite under anaerobic conditions at an intensity of 150 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} of 650 nm light and the results are shown in Fig. 2. The phase lag increases as nitrite is added with the largest change occurring between 1 and 6 mM. It would appear that at 25 mM, nitrite has reached a saturating level. Similarly the rate of oxygen evolution increases as the nitrite level is raised, with 25 mM again being saturating. As in the previous experiment the phase in oxygen is 5° larger than the largest value observed with nitrite while the oxygen yield under aerobic conditions is smaller than the value observed in the presence of nitrite.

The cells used in this study were normally cultured in a Warburg-Buck medium in which the source of nitrogen was 25 mM potassium nitrate. However cells were grown on Warburg-Buck medium in which the only source of nitrate was 25 mM ammonium chloride. Such cells were investigated in the modulated electrode and gave similar responses to transitions between aerobic and anaerobic media. But when sodium nitrite (25 mM) was added to an anaerobic medium bathing these cells, there was no alteration in either the phase or the rate of oxygen evolution.

Chlorella cells which had been dark adapted for 5 min and were immersed

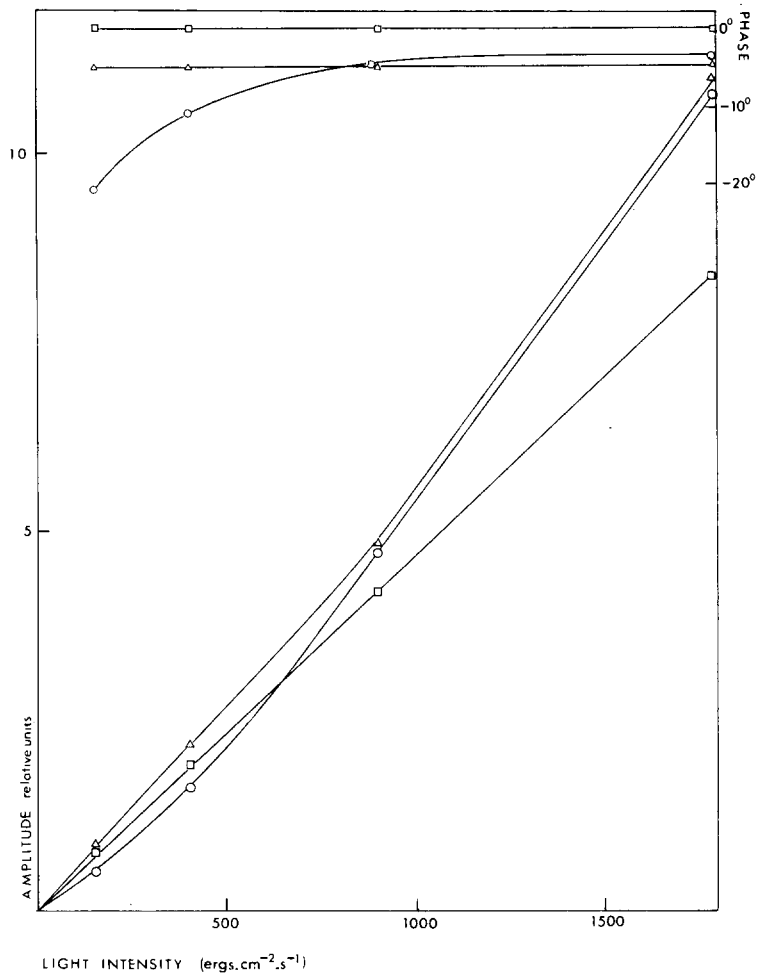

Fig. 1. The relative amplitude (large symbols) and phase lag (small symbols) of the oxygen signal as functions of the mean intensity of the modulated 650 nm light. The sample was a monolayer of *Chlorella vulgaris* cells immersed in 100 mM NaCl plus 10 mM Tris buffer (pH 7.6). This solution was in equilibrium with a gas mixture containing 95% air and 5% carbon dioxide (squares) or 95% nitrogen and 5% carbon dioxide (circles and triangles). 25 mM sodium nitrite was also present in the experiment described by the triangles. The phase angle was arbitrarily set at zero for the solution in equilibrium with the air-CO_2 mixture. The temperature was 21°C and the modulation frequency was 16 Hz.

in an aerobic solution, gave the normal damped oscillating pattern of oxygen pulses in response to a series of saturating light flashes at intervals of 0.3 s (Fig. 3c). When the Kok model [7] was fitted to these results the best fitting values of the parameter were $\alpha = 0.3$, $\beta = 0.11$, the initial value of $S_1/S_0 = 3.0$. Under anaerobic conditions, the above mentioned pattern of oxygen pulses disappeared and was replaced by that shown in Fig. 3b. The steady state value of the oxygen pulses was usually about 70% of the aerobic value. The best fitting values were $\alpha = 0.86$, $\beta = 0.11$, $S_1/S_0 = 3.0$ and thus only α charged to any significant extent.

On adding 1 mM sodium nitrite to an anaerobic solution, the steady state

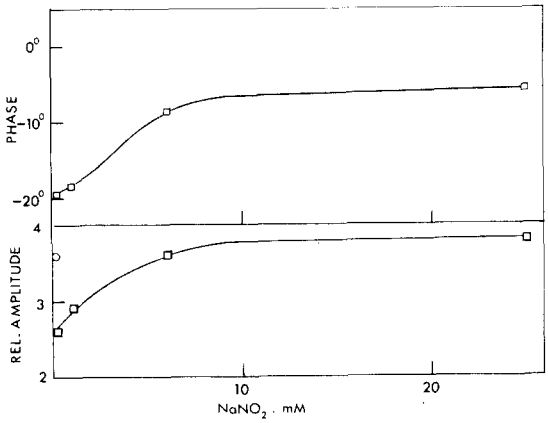

Fig. 2. The relative amplitude and phase lag of the oxygen signal as functions of the concentration of sodium nitrite added to the bathing medium which was in equilibrium with a gas mixture containing 95% nitrogen and 5% carbon dioxide. The mean light intensity was 150 ergs · cm^{-2} · s^{-1} of 650 nm light. The other conditions were as described in Fig. 1. The relative values of the phase lag and amplitude found with an aerobic bathing medium are indicated by zero degrees and the circular symbol, respectively.

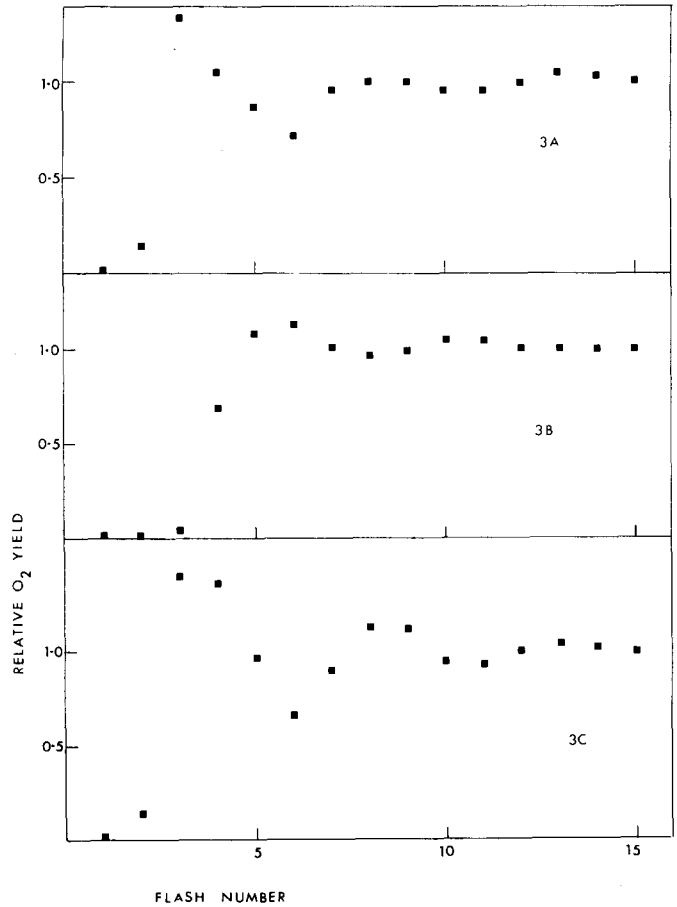

Fig. 3. The relative oxygen yields from a monolayer of *Chlorella* cells exposed to a series of saturating light flashes spaced 0.3 s apart. The *Chlorella* were kept in darkness for 5 min prior to each series of flashes. A bathing medium in equilibrium with 95% air and 5% carbon dioxide was used for the results described in Fig. 3c; a medium in equilibrium with 95% nitrogen and 5% carbon dioxide was used for the results described in Figs. 3b and 3a; 1 mM sodium nitrite was also present in the medium when the results described in Fig. 3a were obtained. The results were normalized so that the steady state yield was unity in all three experiments. Other conditions were as described in Fig. 1.

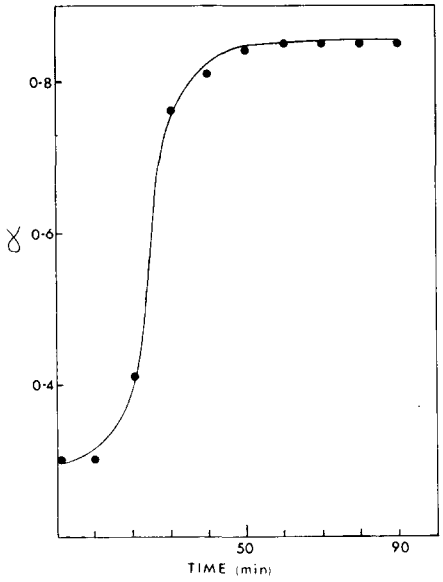

Fig. 4. The change in the best fitting value of the parameter α describing the numbers of misses in the Kok model of Photosystem I [7]. At time zero the medium was changed from one in equilibrium with a gas mixture containing 95% air and 5% carbon dioxide to one in equilibrium with a mixture of 95% nitrogen and 5% carbon dioxide. The cells were kept in darkness except for a series of fifteen saturating flashes delivered every 10 min to permit the evaluation of α.

oxygen yield rapidly increased was later accompanied by the restoration of the normal damped oscillations. After about 10 min exposure to the nitrite the best fitting values were $\alpha = 0.3$, $\beta = 0.11$, the initial value of $S_1/S_0 = 3.0$ (see Fig. 3a). Sodium nitrite was used at 1 mM because it was found that higher concentrations (e.g., 25 mM) could lead to an inhibition of the oxygen yield in these experiments.

The time course of the increase in value of α which followed the removal of oxygen from the medium is shown in Fig. 4. It can be seen that the transition takes about 30 min after which a stable value of α is obtained.

The deactivation of the S_2 state in the dark was also monitored and the results are shown in Fig. 5. In the presence of an air equilibrated solution the value of S_2 falls to 50% of its original value in about 10 s, while in an anaerobic medium this requires only 5 s. The addition of 1 mM sodium nitrite to an anaerobic medium slows the decay of the S_2 state so that approx. 17.5 s elapse prior to the S_2 concentration reading 50% of its original value.

Discussion

It has been shown that at low light intensities the addition of 25 mM sodium nitrite to an anaerobic medium can partly eliminate the effects caused by anaerobiosis, i.e. it leads to increases in both the phase lag and the oxygen yield. According to the model proposed by Sinclair et al. [4] this would imply that nitrite suppresses the backflow of electrons from between the two photosystems to the oxidizing side of Photosystem II. This could mean that nitrite

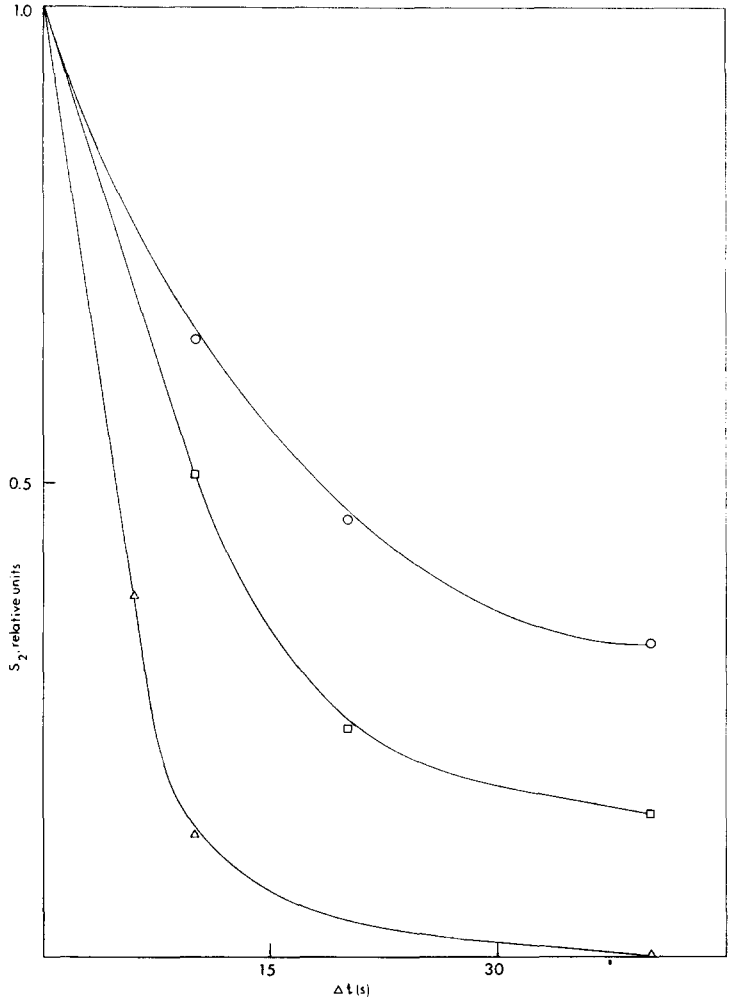

Fig. 5. The deactivation of the S_2 state of the charge storage complex of Photosystem II (see Kok et al. [7]) as a function of the dark period. A dark adapted monolayer of *Chlorella* cells was exposed to a single saturating light flash followed by a dark period (Δt) and then given two more saturating flashes spaced 0.3 s apart. The yield of the third flash denotes the relative number of charge storage complexes in the S_2 state after Δt s. The square symbols represent results obtained in the presence of oxygen, the triangles represent results obtained in the absence of oxygen and the circles, results obtained in the absence of oxygen but in the presence of 1 mM sodium nitrite.

interacts directly with an intersystem redox pool or suppresses cyclic electron transport around Photosystem I by accepting electrons from Photosystem I. It is therefore of interest that Losada et al. [8] showed that *Chlorella* could reduce nitrite in a process which was light stimulated. Also Zumft et al. [9] isolated the enzyme nitrite reductase from *Chlorella* and showed that it would catalyze the reduction of nitrite to ammonia only if reduced ferredoxin were available. Neither reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate could replace reduced ferredoxin. Thus these studies make it seem probable that nitrite has its effect by

suppressing cyclic electron flow around Photosystem I which leads to the oxidation of the relevant intersystem redox pools which in turn decreases the backflow of electron around Photosystem II.

It was also noted that nitrite has less and less effect on photosynthesis under anaerobic conditions as the light intensity is increased so that the phase lag and relative rate of oxygen evolution at 1800 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} are unaffected by its presence. These findings can be related to the work of Hattori [10] who studied the rate of nitrite reduction in *Anabaena cylindrica*. He observed that under anaerobic conditions the rate of nitrite reduction reached its maximum value at a light intensity which was only about 10% of that required to saturate CO_2 fixation. If such a phenomenon occurs here, then the lack of effect of nitrite at high light intensities could simply reflect the inability of nitrite reductase to catalyse the reduction of nitrite at a rate which was comparable with the rate of photosynthetic electron transport. Similarly the dependence of the phase lag and the rate of oxygen evolution on nitrite concentration in the medium (Fig. 2) may well reflect the rate at which nitrite is entering the cells and thus variations in the internal concentration of nitrite.

The ability of nitrite to increase the phase lag and the oxygen yield at low light intensities and in anaerobic conditions was absent with cells which had been grown with ammonium chloride as their source of nitrogen. This result is consistent with the work of Losada et al. [8] who showed that the enzyme nitrite reductase was repressed in *Chlorella* cells grown in a medium containing only ammonium ions as the source of nitrogen. If there is no nitrite reductase in the cells used in these experiments there can be no reduction of nitrite to oxidize ferredoxin and so nitrite will have no effect on the phase lag or oxygen yield.

The failure of sodium nitrate to alter the phase lag or the oxygen yield may be due to the properties of the enzyme nitrate reductase. This enzyme when studied in vitro displays between 1/5 and 1/20 the activity of nitrite reductase, which presumably explains the low concentration of nitrite found in vivo (Hewitt et al. [11]). Thus when nitrate (25 mM) is provided in these experiments it neither has any effect directly nor due to the relative inefficiency of nitrate reductase does it appear to increase the chloroplast concentration of nitrite significantly.

The experiment with glucose was designed to test the idea that although the *Chlorella* were immersed in an anaerobic medium the concentration of oxygen within the cell might still be significant. The addition of glucose was intended to stimulate respiration and thus lower the intracellular oxygen concentration which should then lead to a decrease in both the phase lag and rate of oxygen evolution. As the addition of glucose did lead to both of these effects, it seems likely that the intracellular concentration of oxygen is not zero even when the cells are in an anaerobic solution.

The removal of oxygen from the bathing medium had a marked effect on the flash yield pattern (Fig. 3). The maximum yield was found on the sixth flash rather than the third flash and there was a large increase in the value of α from 0.3 to 0.86. Bouges [12] saw a similar delay in the maximum flash yield in *Chlorella* in the presence of low concentrations of hydroxylamine. She attributed this to a reduction of the charge storing complex S, by the hydroxyl-

amine which was alleviated by the light. This corresponds nicely with the model we have proposed (Sinclair et al. [4]) in which the removal of oxygen causes an increased backflow of electrons to the oxidizing side of Photosystem II. This provides a reducing environment for the charge storing complex and presumably hinders the production of S_4 stage which is the necessary precursor of oxygen evolution. The presence of nitrite alleviates this situation as the findings with the modulated electrode would indicate by reducing the backflow of electrons.

The influence of the presence or absence of oxygen on the deactivation of the S_2 state was shown in Fig. 5. These results are similar to those obtained earlier by Diner [13]. Once again the presence of nitrite reverses the effects of anaerobiosis and slows down the deactivation process indicating that the reduction of nitrite by Photosystem I can alter events on the oxidizing side of Photosystem II.

There was a significant difference in the sensitivity which *Chlorella* displayed to nitrite in the deactivation experiments where 25 mM nitrite caused inhibitory effects as compared with the experiments in the modulated polarograph where 25 mM was routinely used with no problem. This difference may may reflect an adverse effect which nitrite has only in the dark since the *Chlorella* in the deactivation experiments spent almost the whole time in the dark. In the light the nitrite concentration in the cell will be kept lower than that in the surrounding medium due to its continual reduction. Apart from the inhibitory effect, it only required 1 mM nitrite to completely eliminate the effects of anaerobiosis on the deactivation of the S_2 state but 25 mM to partly reverse the effect on the phase lag which we believe is due to the speeding up of the $S_4 \rightarrow S_3$ back reaction in the presence of sufficient oxygen. We would suggest that this may reflect the differing amounts of positive charge associated with the two S states. The S_4 state should react much more readily with an electron donor than the S_2 state. Thus the supply of electron donors must be reduced much more drastically (which will require a higher nitrite concentration) to reduce the rate of the $S_4 \rightarrow S_3$ reaction than the rate of the $S_2 \rightarrow S_1$ reaction.

In conclusion it has been shown that under anaerobic conditions there is a back flow of electrons to the oxidizing side of Photosystem II. These electrons can speed up the back reaction $S_4 \rightarrow S_3$ as evidenced by the modulated polarograph experiments and also the deactivation of the S_2 state. These two effects can be reversed either in part or in full by the addition of a suitable concentration of nitrite to the anaerobic medium which probably suppresses cyclic electron transport.

Acknowledgements

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