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THE INFLUENCE OF ANIONS ON OXYGEN EVOLUTION BY ISOLATED SPINACH CHLOROPLASTS

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A study has been made of the onset of chloride deprivation on the oxygen-evolving characteristics of isolated spinach chloroplasts. Using a modulated oxygen electrode it is found that the type of inhibition depends on the anion replacing chloride in the bathing medium. With nitrate a large increase in phase lag accompanies a relatively small inhibition which can be shown to be consistent with a decrease in the rate constant of the reaction which limits the rate of electron transport between water and Photosystem II. With sulphate there is a very small phase change but a larger inhibition which suggests that replacing chloride with sulphate in an electron-transport chain shuts off that chain. With acetate there is a moderate increase in phase lag and the largest inhibitory effect. The phase-lag increase suggests that acetate is affecting the same chloride-sensitive site as nitrate. However, the inhibition cannot be explained by this effect alone and points to the existence of a second chloride-sensitive site. Of the four forward reactions associated with the Kok model of oxygen evolution (Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475) only $S_3^* \rightarrow S_0$ is slowed down when chloride is replaced by nitrate. This reaction is not slowed down by replacing chloride with sulphate.

Introduction

The early work of Warburg and Luttgens [1] indicated that maximal rates of electron transport in chloroplasts could only be attained if chloride ions were available to these organelles. Our knowledge of this chloride requirement was considerably advanced in a series of papers by Hind and coworkers [2–5]. This group introduced the term 'chloride effect' by which they meant the ratio of the rate of electron transport observed in the presence of chloride to that observed in the absence of chloride. Hind et al. [2] demonstrated that large chloride effects could be produced if chloroplasts were exposed to alkaline media, brief periods at elevated temperatures or uncouplers. More recently a substantial study by Theg and Homann [6] investigated the retention of chloride ions by chloroplast membranes and these workers were able to offer explanations for some of the results obtained by Hind's group. A feature common to the work of Theg and Homann [6] and Hind et al. [2] was that they endeavoured to maximize the chloride effect by pretreating the chloroplasts in some way (e.g., with an uncoupler) in a chloride-free medium. They then measured the rate of electron transport in the absence of chloride and again after chloride was restored to the medium. Thus they observed the effect of restoring chloride to the chloroplasts. The present study took an approach which was different from this. The chloroplasts were not pretreated and were usually observed in a chloride containing medium initially and then in a chloride free medium. It was there-

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fore the onset of chloride deprivation which was observed. This simpler approach did not yield such large chloride effects but did provide new information about the chloride-sensitive parts of the electron transport chain.

Methods

Broken chloroplasts were prepared from 3-week-old, greenhouse grown spinach after the method described by Arnason and Sinclair [7]. The chloroplasts were stored prior to use in 0.33 M sorbitol, 30 mM Mops (3-(*N*-morpholino)propanesulphonic acid) buffer, pH 7.9 and 1 mM $MgCl_2$. The chlorophyll content was measured by the method of Vernon [8] and adjusted to the values indicated below.

The relative rate of oxygen evolution was monitored with a modulated oxygen electrode as described in Sinclair and Arnason [9]. A sample of the chloroplast suspension (30 μg chlorophyll per ml) enriched with a saturating amount of ferredoxin (Sigma Chemical Co., type III) was injected into the electrode assembly. The solution flowing through the assembly contained 0.33 M sorbitol, 30 mM Mops pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM NADP (nicotinamide adenine dinucleotide phosphate). The chloroplasts were left for 10 min in the dark and then illuminated with a light which was modulated at 16 Hz and had been passed through a 640 nm interference filter. The mean intensity of the beam was typically 5.4 $\mu E \cdot m^{-2} \cdot s^{-1}$. All experiments were done in the linear range of rate of O_2 evolution versus light intensity. After about 20 min the O_2 signal had attained a stable value and one of two procedures was followed. Either the same solution was kept flowing for a further 40 min as a control or the solution was altered to a chloride-free medium. In the latter case, an initial 25 ml of chloride-free medium which lacked NADP was passed through the oxygen electrode and then discarded. This was intended to rinse out any chloride ions in the apparatus. The brief absence of NADP during this period did not affect oxygen evolution. Subsequently the normal chloride-free medium with NADP was used. The chloroplasts were observed for a total of 40 min in the chloride-free medium as this was the time for which the rate of O_2 evolution in the control usually remained constant. The phase of the oxygen signal in control experiments always remained constant to $\pm 1°$.

The responses of dark adapted chloroplasts to sequences of brief, saturating light flashes were examined. The experimental arrangement was the same as that described in Arnason and Sinclair [7] except that General Electric FT230 flash tubes were used. The protocol involved immersing the chloroplasts in either a chloride containing experimental medium (0.33 M sorbiton/30 mM Mops, pH 7.9/50 mM NaCl/1 mM $MgCl_2$/1 mM NADP) or one in which another anion replaced the chloride. The chloroplasts were subjected to sequences of 30 saturating light flashes spaced at 0.3 s following each 10 min of darkness until the pattern of oxygen flash yields stabilized. Then the time-course of one of the four forward reactions of the Kok model of O_2 evolution (Kok et al. [10]) was investigated by methods similar to those used by Bouges-Bocquet [11]. Using the nomenclature developed by the latter author, γ_n is the function which measures the probability that an oxygen evolving centre having entered an excited state S_n^* at time zero will have relaxed to a state S_{n+1} after time Δt. γ_0, γ_1, γ_2 and γ_3 were all followed and the only significant difference in protocol from that used by Bouges-Bocquet [11] was that the following expression was used for γ_3:

$$\gamma_3 = \frac{\gamma_7(\Delta t) - \gamma_7(0)}{\gamma_7(\infty) - \gamma_7(0)}$$

where $\gamma_7(\Delta t) = O_2$ yield of flash 7 given Δt s after the preceding flash. $\gamma_7(\infty) = O_2$ yield of flash 7 given 0.3 s after the preceding flash.

This expression lacks two additional small terms dependent on γ_1 and γ_2 but for the arguments presented here these terms are unnecessary and the above expression is adequate.

Results

The response of chloroplasts to chloride deprivation as observed with the modulated oxygen electrode is shown in Figs. 1 and 2. Prior to time zero in these figures, the chloroplasts had been in a chloride medium for a time period which was sufficient to permit them to attain a steady rate of

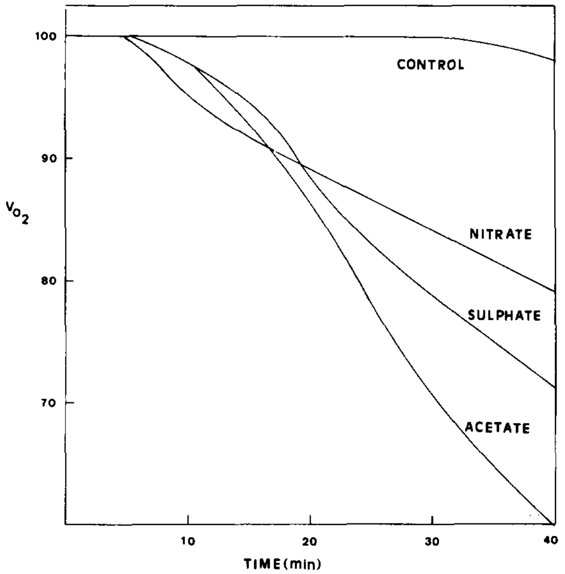


Fig. 1. The relative rate of oxygen evolution (V_{O_2}) from spinach chloroplasts in media containing different anions. The control medium contained 0.33 M sorbitol/30 mM Mops, pH 7.9/1 mM NADP/50 mM NaCl/10 mM $MgCl_2$. The other media were identical except that the chloride ions were replaced by the indicated anions. The chloroplasts were illuminated with 5.4 $\mu E \cdot m^{-2} \cdot s^{-1}$ of 640 nm light modulated at 16 Hz. The temperature was 23°C. Prior to time zero the chloroplasts were in a control medium in the modulated oxygen electrode long enough for a steady rate of photosynthesis to be established. At time zero the medium flowing through the apparatus was altered to the one indicated.

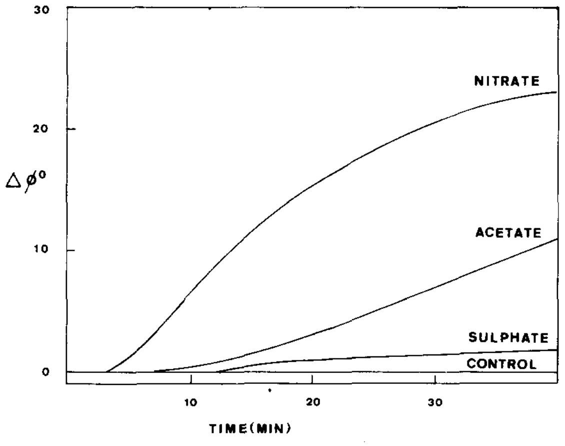
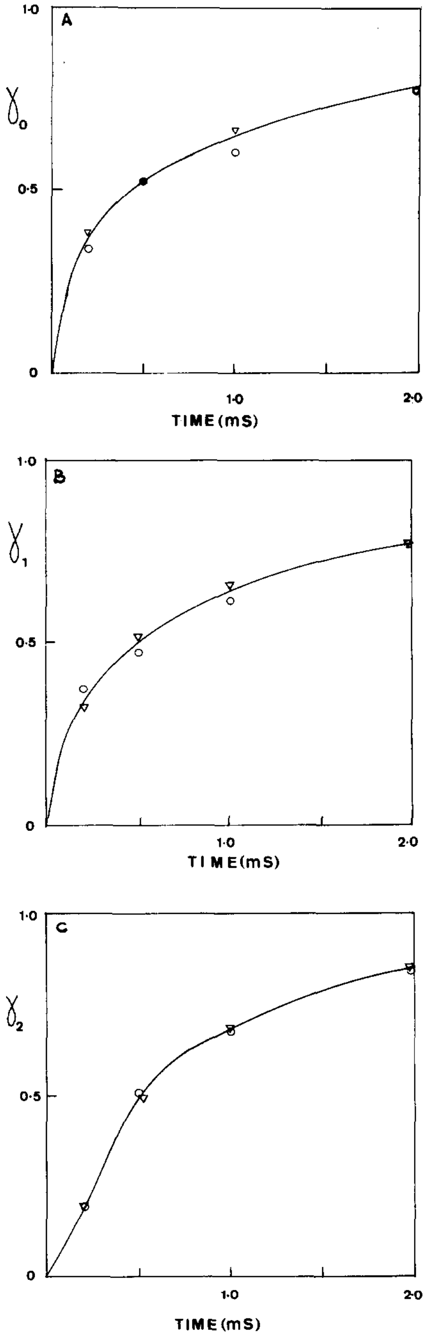


Fig. 2. Changes in phase angle ($\Delta\phi$) of the oxygen signal from spinach chloroplasts in media containing different anions. These results were from the experiments shown in Fig. 1. The phase angle in the control medium has arbitrarily been set at zero.

oxygen evolution. At time zero, their bathing medium was altered to one of the chloride free media indicated in the figures. For about 5 min there was no change in the behaviour of the chloroplasts and this presumably represents the time required to create the start of chloride deficiency in the chloroplasts. Thereafter an inhibition of the rate of oxygen evolution appeared (Fig. 1) which increased with time. Initially this inhibition developed most rapidly in the nitrate medium but subsequently the inhibition grew more rapidly in the acetate and sulphate media. At the end of 40 min the inhibition of the rate of oxygen evolution was 21% in the nitrate medium, 29% in the sulphate medium and 40% in the acetate medium. Concurrently increases in the phase lag appeared with the largest change being manifested in the nitrate medium (23°) with lesser increases in acetate (11°) and sulphate (2°). When chloroplasts were exposed to either nitrate, acetate or sulphate in the presence of chloride, none of the above responses were observed thus the absence of chloride is a necessary condition of these experiments.

The progress of the four forward reactions of the Kok model of oxygen evolution was followed with chloroplasts immersed in both chloride and nitrate media. The results are shown in Figs. 3, A–D. It is apparent that to a close approximation the time-courses of γ_0, γ_1 and γ_2 are unaffected by the choice of anion. The half-times for these reactions are similar to those found by Bouges-Bocquet [11]. However, it is also apparent that γ_3 is sensitive to the choice of chloride or nitrate, being considerably slower to increase when the nitrate medium is used. The course of γ_3 in the presence of chloride can be described by a simple exponential and the half-time of yields a rate constant of 330 s^{-1}. Treating the nitrate results in a similar fashion gives a rate constant of 155 s^{-1}. Thus only one of the four forward reactions is sensitive to the substitution of nitrate for chloride. Experiments were also performed to investigate the effect upon γ_3 of substituting sulphate for chloride. The results are shown in Fig. 3D and the time course of γ_3 in the presence of sulphate is very similar to that in the presence of chloride and quite different from the results with nitrate.

In the preceding experiments when a chloride medium was used after a chloride-free medium,



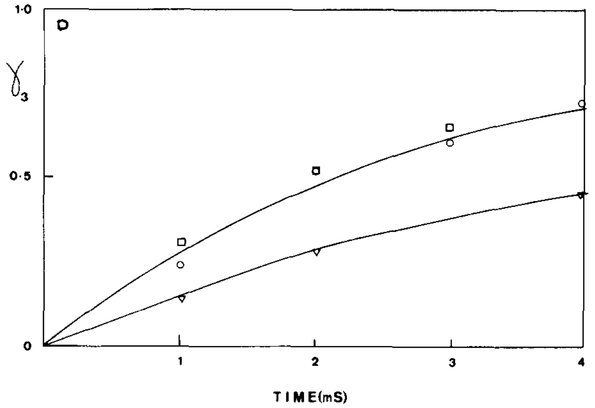


Fig. 3. The time-courses of the forward reactions of the Kok scheme in chloride and nitrate media. (A) $S_0^* \rightarrow S_1$. The chloroplasts were in darkness for 5 min, given three flashes of light separated by 0.3 s intervals, followed by 6 min of darkness then 30 flashes at 0.3 s intervals except for the first and second flashes which were separated by Δt.

$$\gamma_0 = \frac{Y_4(\Delta t) + Y_3(\Delta t) - Y_3(0.3\ \mathrm{s})}{Y_4(0.3\ \mathrm{s})}$$

(B) $S_1^* \rightarrow S_2$. The chloroplast were left in the dark for 10 min and given 30 flashes at 0.3 s intervals except for the first and second flashes which were separated by Δt.

$$\gamma_1 = \frac{Y_3(\Delta t)}{Y_3(0.3\ \mathrm{s})}$$

(C) $S_2^* \rightarrow S_3$. The chloroplasts were left in the dark for 10 min and given 30 flashes at 0.3 s intervals except for the second and third flashes which were separated by Δt.

$$\gamma_2 = \frac{Y_3(\Delta t)}{Y_3(0.3\ \mathrm{s})}$$

(D) $S_3^* \rightarrow S_4$. The solutions used had 0.1 mM potassium ferricyanide added to them. The chloroplasts were left in the dark for 10 min and given 30 light flashes separated by 0.3 s except for the third and fourth flashes which were separated by Δt.

$$\gamma_3 = \frac{Y_7(\Delta t) - Y_6(0.3\ \mathrm{s})}{Y_7(0.3\ \mathrm{s}) - Y_6(0.3\ \mathrm{s})}$$

The circles represent the observations in a chloride medium which contained 0.33 M sorbitol/30 mM Mops, pH 7.9/1 mM NADP/50 mM NaCl/1 mM $MgCl_2$. The triangles are results with a nitrate medium and the squares with a sulphate medium which were identical to the chloride medium except that the chloride was replaced by either nitrate or sulphate. All experiments were done at 23°C.

there was a very significant increase in the steady-state flash yield, demonstrating the presence of a chloride effect.

Discussion

The three anions chosen as replacements for chloride gave rise to three distinct patterns of inhibition as shown in figs. 1 and 2. An appreciation of the significance of these differences can best be gained by considering the analysis of the operation of the modulated electrode presented by Joliot et al. [12]. These workers demonstrated that the phase lag of the oxygen signal depended on the diffusion of oxygen from the chloroplasts to the electrode and the rate constant (k) of the reaction which rate limits electron transport between Photosystem II and the water-splitting act. As it is unlikely that merely substituting one anion for another is going to alter oxygen diffusion, the observed phase changes must be explained in terms of variations in the value of k. This can be done using mathematical relationships developed by Joliot et al. [12] and a value for k in the initial chloride medium. Since a value for k is not available for the experimental conditions used here a special procedure has been adopted. A value of k is assumed to characterize the situation in the chloride medium and a value of the phase lag due to this is calculated. To this phase lag are added the changes in phase lag observed in the nitrate medium at different times. The values of the rate constant for these nitrate phase lags are computed and then used to calculate the expected relative rates of oxygen evolution which are then compared with the observed values. The relationships used are from the work of Joliot et al. [12].

$$\tan \phi = w/k \qquad (1)$$

$$V_{O_2} = A \cdot \frac{k}{\sqrt{k^2 + w^2}} \qquad (2)$$

where w = angular velocity, ϕ = phase lag due to rate limiting reaction, A = proportionality constant, V_{O_2} = relative rate of oxygen evolution.

The results of these calculations for various times during the nitrate experiment and three different values of k in the chloride medium are shown in Table I where they are compared with the observed values. It can be seen that for $k = 225$ s^{-1}, the calculated rates of O_2 evolution are consistently lower than the observed values whereas for $k = 425$ s^{-1} the calculated values are consistently higher. For $k = 325$ s^{-1} the calculated values are in close agreement with the observed values and thus it is possible to relate the observed phase lag changes and inhibition of O_2 evolution with this value of k. The only value of k measured with the modulated oxygen electrode for chloroplasts is 215 ± 20 s^{-1} (Arnason and Sinclair [13]) but this was measured under different conditions.

TABLE I

THE RESPONSE OF THE RATE OF OXYGEN EVOLUTION TO A CHLORIDE-FREE, NITRATE MEDIUM: A COMPARISON OF OBSERVED AND CALCULATED VALUES

Phase changes observed with chloroplasts in a nitrate medium. Data taken from Fig. 2. V_{calc}, calculated value of rate of oxygen evolution in a nitrate medium expressed as a percentage of the rate in a chloride medium at time zero. The value of k assumed is shown at the top of each column. The method of calculation is described in the Discussion. V_{O_2}, observed rate of oxygen evolution in the nitrate medium as a percentage of the rate in the chloride medium at time zero. Data taken from Fig. 1.

Time		V_{calc} for k			V_{O_2}
		225 s^{-1}	425 s^{-1}	325 s^{-1}	
10 min	6.5°	94.7%	97%	96%	95%
20 min	15.5°	84.6%	90%	88%	89%
30 min	20.5°	78%	85%	83%	83.5%
40 min	23°	75%	83%	80.2%	79%

It can be concluded from this calculation that the inhibition and increase in phase lag observed in a nitrate medium are consistent with there being a decrease in the rate constant of the reaction which limits the rate of electron transport on the oxidizing side of Photosystem II when nitrate replaces chloride.

The results with sulphate revealed that essentially no phase change accompanies the inhibition. Such a result is consistent with the idea that when chloride is replaced in an electron transport chain by sulphate, that chain ceases to function. In contrast, acetate did give rise to a significant increase in phase lag which is consistent with acetate affecting the same site as nitrate. However, the extent of the inhibition observed with acetate was far too large to be explained by the increased phase lag. The simplest explanation of this discrepancy is that there is a second site where chloride is required, which is also inhibited when acetate replaces chloride. The observed inhibition would be largely ascribed to this second site while the increase in phase lag would be ascribed to the first. Evidence which supports this hypothesis of two functional sites for chloride comes from the work of Critchley et al. [14]. These workers found that there were two binding sites for chloride in the stacked thylakoid region of spinach chloroplasts, which is where Photosystem II is thought to operate (Åkerlund et al. [15]).

The results of the experiments using sequences of saturating light flashes demonstrated that of the four parameters γ_0, γ_1, γ_2 and γ_3 only the last was significantly changed when observed in a nitrate medium as compared with a chloride medium. This parameter γ_3 characterises the slowest of the four S state transitions which is also the one which leads directly to oxygen evolution. It is therefore the parameter which should be most closely related to the rate constant of the rate limiting reaction which is involved in determining the phase lag observed in the modulated oxygen electrode studies. Thus the flash experiments are in good agreement with modulated electrode experiments in locating a chloride sensitive site close to the oxygen evolving act. The failure of sulphate to change the time course of γ_3 is consistent with the conclusions drawn from the experiments with the modulated electrode. The latter suggested that when sulphate replaced chloride in an electron-transport chain, that chain became inoperative. Thus the only chains which could be observed would be those which still had chloride present and so exhibited the normal kinetics which is what was observed.

In conclusion, the findings presented here show that there is a chloride requiring site involved in the $S_3^* \rightarrow S_0$ transition and a second chloride-requiring site at some other location on the electron-transport chain.

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