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Changes in thylakoid behaviour in chloride-free media

John Sinclair *

Biology Department, Carleton University, 1125 Colonel By drive, Ottawa, Ontario K1S 5B6, Canada

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Abstract

The photosynthetic activity of spinach thylakoids has been monitored under light-limiting conditions with a modulated oxygen electrode. At pH 7.9, replacing chloride with sulphate in the bathing medium causes a larger inhibition of oxygen evolution if the solution change is performed in the dark, but replacing the chloride with nitrate causes an inhibition which is unaffected by the illumination conditions. At pH 6.5, replacing chloride with sulphate does not inhibit oxygen evolution but replacing chloride with nitrate does. These findings show that sulphate but not nitrate conforms to the chloride replacement mechanism of Theg and Homann [1]. Returning chloride to thylakoids which have been inhibited with nitrate restores the original phase angle and a large part of the lost oxygen evolution. Thus the electron transport chains which respond to the chloride restoration do not appear to have been structurally damaged. Thylakoids which have been inhibited with a sulphate medium do not respond to the restoration of a chloride medium. At pH 7.9, the variable fluorescence observed after dialysis against sulphate or nitrate media and in the presence of DCMU rises slowly compared to a chloride control and there is a large increase in the complementary area but no changes in F_o or F_{max}. The fluorescence changes can be abolished under conditions which permit the inhibition of oxygen evolution and thus may represent a separate chloride-sensitive process.

Key words: Chloride; Photosystem II; Thylakoid; Oxygen evolution; Fluorescence induction

1. Introduction

The removal of chloride ions from thylakoid membranes decreases the rate of electron transport between the water-splitting site and Photosystem II [2–4]. Substitution of other anions for chloride lowers the efficiency of electron transport [2,5]. It has been estimated that there are 5 chloride ions present for each reaction centre in Photosystem II [1] and studies of the exchange of chloride ions have suggested that one chloride ion per P680 exchanges in the light with a half-time of several hours [6] and that there is a class of tightly bound chloride ions which exchange very rapidly [7]. The site of action of chloride and its role within Photosystem II are uncertain but have been frequently associated with the manganese cluster (e.g., [8–10]). Others (e.g., [11]) have stressed that chloride helps to maintain the structural integrity of the Photosystem. A variant of this last idea is that chloride only stimulates Photosystem II function after the Photosystem has been partly denatured [12].

The objective of the present study was to improve our knowledge of the changes in Photosystem II function which occur as a result of chloride depletion. To this end, two very sensitive monitors of Photosystem II activity in spinach thylakoids were observed, the fluorescence induction curve measured in the presence of DCMU and the relative rate of oxygen evolution. The latter parameter was followed with a modulated oxygen electrode [13] in which thylakoids are illuminated with light whose intensity is modulated in an approximately sinusoidal fashion, and the subsequent modulations in oxygen concentration are detected. The amplitude of electric current produced by these oxygen modulations at the platinum electrode is proportional to the mean rate of oxygen evolution but does not provide an abso-

* Corresponding author. Fax: + 1 (613) 7884497.

Abbreviations: S_n, the state of the charge storage complex in Photosystem II bearing n positive charges; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; F_o, initial level of fluorescence; F_v, variable fluorescence; LED, light-emitting diode; MOPS, 3-[*N*-morpholino]propanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; P680, reaction centre of Photosystem II; k_{et}, rate constant of reaction limiting rate of electron transport between water-splitting act and Photosystem II.

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lute value of the rate of oxygen evolution. The phase of this current is determined by the diffusion of oxygen to the electrode and k_{et}, the rate constant for the reaction which limits the rate of electron transport between the water-splitting act and P680 [13]. A solution flows through the modulated oxygen electrode and exchanges molecules through a dialysis membrane with the compartment containing the thylakoids. By changing the composition of this solution it is possible to remove chloride ions from the environment of the thylakoids. In an earlier study with this device, it was shown that replacing chloride with sulphate results in an inhibition of oxygen evolution but no significant phase change, while a nitrate medium causes both an inhibition and a large phase change [14]. A study of oxygen flash yields revealed that the $S_3 \rightarrow S_0$ transition of the water-splitting complex [15] was slower in a nitrate medium than a chloride medium, but this was not true for a sulphate medium [14]. It was proposed that nitrate has a specific effect at a chloride-requiring site associated with the $S_3 \rightarrow S_0$ transition, while sulphate completely inhibits individual electron transport chains at some unknown site. The product of sulphate inhibition was described in other studies as a modified S_2 state [16], but more recently as a modified form of the S_3 state [17] or an (S_2 + radical) state [18].

Our knowledge of the chloride effect in thylakoids has to a large extent been obtained with uncoupler-treated thylakoids immersed in sulphate media and observed under light-saturating conditions. Since restricting observations to such limited circumstances is unlikely to reveal the full characteristics of chloride depletion, the thylakoids employed here were not treated with uncouplers and were observed under light-limiting conditions. A major aspect of this study was the contrast between the results obtained with sulphate and nitrate as alternative replacement ions for chloride.

2. Materials and methods

Chloroplasts were isolated from greenhouse-grown spinach by the method of Jensen and Bassham [19] as modified by Heber [20]. Chlorophyll concentrations were measured by the method of Vernon [21]. Prior to use, chloroplasts were broken by immersion for 30 s in buffer (30 mM MOPS buffer (pH 7.9)).

The modulated oxygen electrode used here was described in Sinclair and Arnason [22]. This apparatus is divided into three compartments by two dialysis membranes. A thylakoid suspension (chlorophyll concentration = 150 $\mu g\ ml^{-1}$) mixed with 0.1 mg ml^{-1} ferredoxin (Sigma Chemical Co., type III) was injected into the bottom compartment and allowed to settle on the platinum electrode. The solution flowing through the middle compartment contained 330 mM sorbitol, 30 mM MOPS (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM NADP (Chloride medium). The sulphate and nitrate media contained these anions in place of chloride. The upper compartment was filled with 330 mM sorbitol, 30 mM MOPS (pH 7.9), 0.1 mM NaCl. For experiments performed at pH 6.5, the MOPS in the preceding solutions was replaced MES buffer at pH 6.5. The chloroplasts were illuminated with a light-emitting diode (Hewlett Packard, HLMP 3750). The power supply to the diode was built by the Science and Technology Centre at Carleton University. The current through the LED was modulated at 16 Hz and the mean intensity incident on the chloroplasts was 10 μmol photons $m^{-2}\,s^{-1}$. The oxygen signal size and phase angle were measured by a PAR model 129 lock-in amplifier. It should be noted that the measured phase angle is inversely related to the phase lag so that for example, an increase in phase angle corresponds to a decrease in phase lag. Results were recorded via an A/D board (Data Translation DT2801) by a microcomputer (Raven, Science and Technology Centre, Carleton University). The software used was Asyst, version 2.1 (Keithley Instr. Ltd.)

Observations were made of fluorescence emission from chloroplasts which had been dialysed against chloride or chloride-free media. The media used were identical to those described above except that 0.1 mM methyl viologen replaced the NADP. Samples of broken chloroplasts were injected into the lower of two chambers in a lucite container. The depth of this lower chamber was 150 μm and it was separated by a dialysis membrane from the upper chamber through which a chloride, nitrate or sulphate medium flowed. The chloroplasts were dialysed for 40 min in the dark, then ejected from the apparatus into a fluorescence cuvette. The final volume of the ejected suspension was adjusted to 2 ml with dialysis medium to give a chlorophyll concentration of 8 $\mu g\ ml^{-1}$. The suspension was dark-adapted for 5 min before DCMU was added to a final concentration of 50 μM. After one more minute of darkness the fluorescence induction transient was recorded. Illumination was provided by four light-emitting diodes, each directed against one face of the cuvette. Mean intensity per face = 10 μmol $m^{-2}\,s^{-1}$. The fluorescence induction transients were observed and analysed as described in [23].

3. Results

It has been shown that the presence or absence of light during chloride depletion with sulphate media is important in determining the extent of inhibition of oxygen evolution observed under light-saturating conditions [1]. The experiments illustrated in Figs. 1 and 2

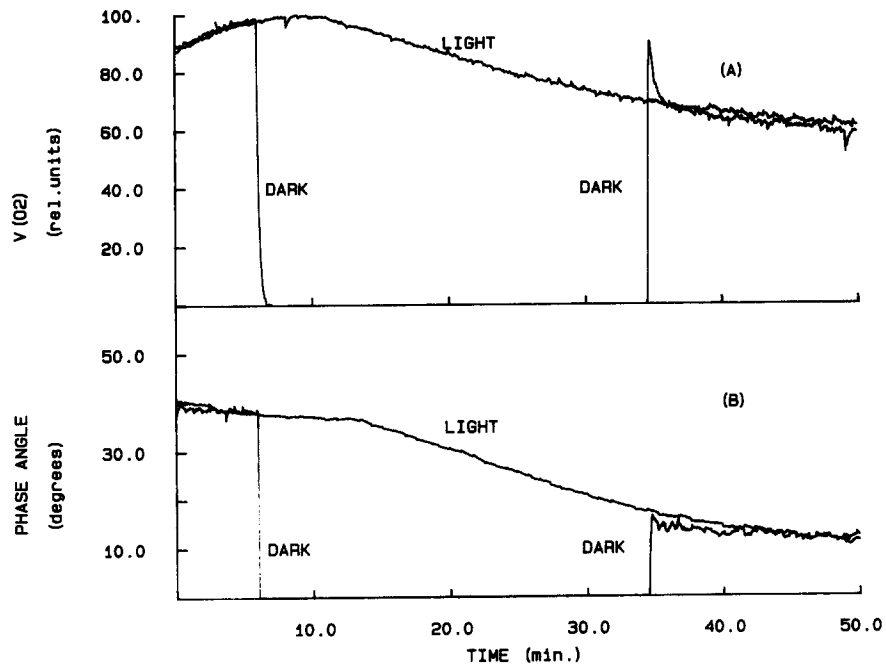

Fig. 1. $V(O_2)$, the relative rate of oxygen evolution (A) and the phase angle (B) versus time for thylakoids which were dialysed initially against a chloride medium and then against a nitrate medium at pH 7.9. It should be noted that a decline in the phase angle corresponds to an increase in the phase lag between the light and oxygen modulations. The results labelled 'Dark' were obtained when the nitrate medium was introduced at the start of a 30-min dark period. In the 'Light' experiment the solution change was performed at the same time as in the 'Dark' experiment but illumination was maintained. Mean light intensity = 10 μmol photons $m^{-2} s^{-1}$. Temperature = 22°C.

investigated if this was also true under light-limiting conditions when nitrate or sulphate was used to replace chloride at pH 7.9. In Fig. 1 the nitrate medium was introduced during continuous illumination and resulted in decreases of about 30% in the rate of oxygen evolution (Fig. 1A) and 28° in the phase (Fig. 1B). When the nitrate medium was introduced at the start of a dark period, the reductions in the rate of oxygen

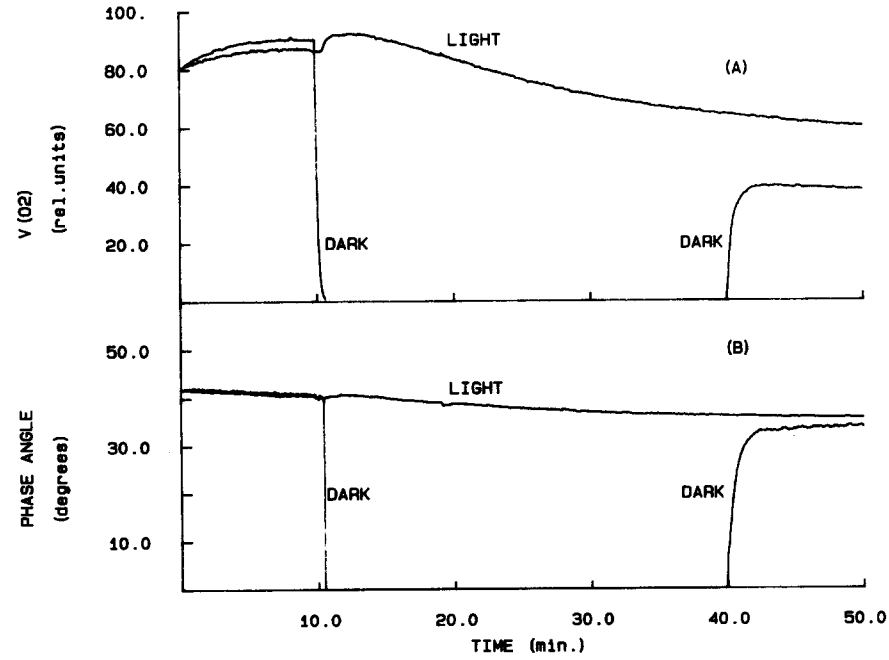

Fig. 2. The $V(O_2)$, the relative rate of oxygen evolution (A) and the phase angle (B) versus time for thylakoids which were initially dialysed against a chloride medium and then a sulphate medium at pH 7.9. Other conditions as described for Fig. 1.

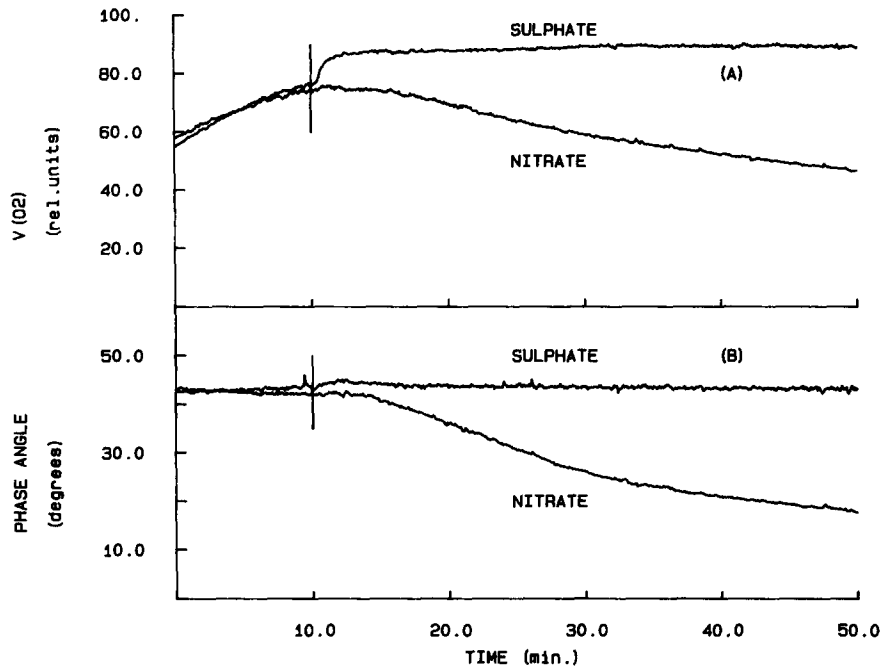

Fig. 3. The relative rate of oxygen evolution (A) and the phase angle (B) as functions of time for thylakoids which were initially dialysed against a chloride medium and then a nitrate or a sulphate medium at pH 6.5. The solution changes began at the vertical bars. Other conditions as described for Fig. 1.

evolution and the phase angle change after the dark period were very similar to those seen with the continuous illumination. The transient in the rate of oxygen evolution found when illumination was restored is the burst [24]. When a sulphate medium replaced the chloride medium in the light, there was a small increase in the rate of oxygen evolution immediately after the solution change (Fig. 2A). The cause of this increase is unknown. Later there was a long fall in the rate of oxygen evolution which was about 30% lower at the

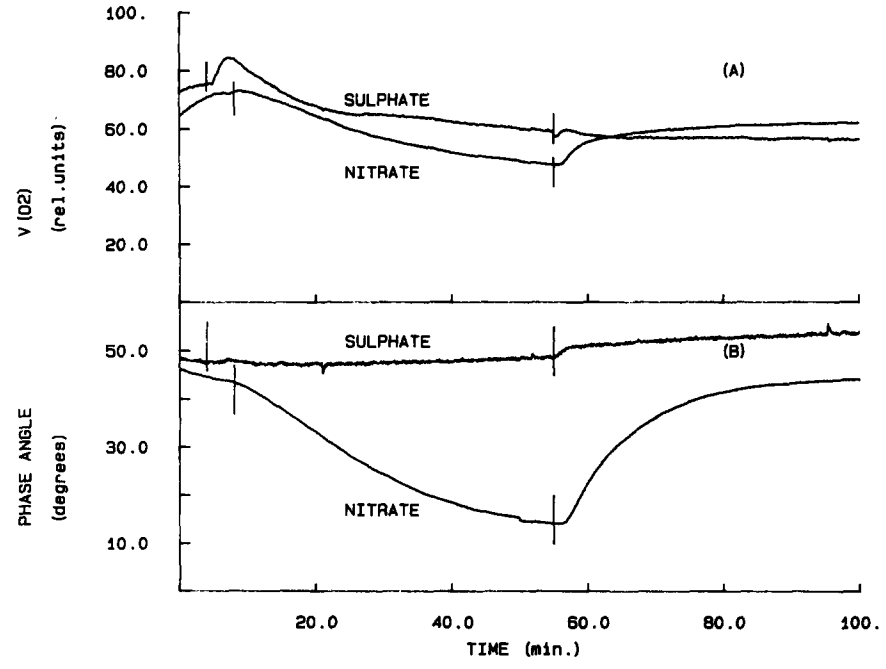

Fig. 4. The relative rate of oxygen evolution (A) and the phase angle (B) as functions of time for thylakoids which were initially dialysed against a chloride medium then, after the first vertical bars, in a nitrate or a sulphate medium. After the second vertical bars, the chloride medium was restored. The experiments were performed at pH 7.9.

end of observations than it had been in the chloride medium. The corresponding dark transition with sulphate resulted in a 60% inhibition in the rate of oxygen evolution (Fig. 2A). Because of the long time constant used in this experiment, the oxygen burst was not detected. There were small phase angle decreases of 5° and 8° with the light and dark transitions respectively (Fig. 2B).

Another feature of the chloride effect seen previously [1] was the absence of inhibition with a sulphate medium at acid pH values unless the thylakoids had been exposed to uncouplers. In the present study, replacing the chloride medium with either the sulphate or nitrate medium at pH 6.5 caused the changes in the rate of oxygen evolution and phase angle shown in Fig. 3. The solution change in both experiments began at 10 min as indicated by the vertical bars. The introduction of the sulphate medium increased the rate of oxygen evolution by 15% which remained constant thereafter (Fig. 3A). The phase angle showed no significant change as a result of the introduction of sulphate (Fig. 3B). After the nitrate medium was introduced the rate of oxygen evolution fell by 40% (Fig. 3A) and the phase angle by 23° (Fig. 3B). Thus there was an inhibitory effect with nitrate but not with sulphate under light-limiting conditions at pH 6.5 without the use of uncouplers.

The reversibilty of the chloride effects with nitrate and sulphate was examined (Fig. 4) at pH 7.9 by exposing thylakoids to a chloride medium initially, then to a sulphate or nitrate medium before the chloride medium was restored. The vertical bars indicate when the solution changes occurred. The restoration of chloride to the sulphate-exposed thylakoids did not cause an increase in oxygen evolution (Fig. 4A) nor a large phase angle change (Fig. 4B). However, the restoration of chloride to the nitrate-inhibited sample did increase both the rate of oxygen evolution and the phase angle. The rate of oxygen evolution in the nitrate medium fell to 65% of the value exhibited in the chloride medium. When the chloride medium was restored, the rate of oxygen evolution reached 86% of the value reached originally in this medium. The phase angle decreased by 30° in the nitrate medium but increased by this amount when chloride was restored.

The rise of variable fluorescence from thylakoids inhibited with DCMU depends on the movement of electrons through P680 and the availability of electron acceptors. It is thus a sensitive indicator of Photosystem II activity. When this parameter was observed (Fig. 5A) with thylakoids dialysed in a sulphate (curve 2) or a nitrate medium (curve 3) at pH 7.9 for 40 min in the dark, it rose more slowly than did the chloride control (curve 1). The results for each medium have been normalised to the maximum value of the variable fluorescence. The complementary area above the fluorescence induction curve can be resolved into four kinetic phases [23] and the areas of these phases were altered by dialysis against sulphate or nitrate media (Table 1A). The area of the α phase was reduced by 50% following dialysis against the sulphate or nitrate media, while the δ phase area and to a lesser extent the Γ phase area were increased. The rate constants of the various phases changed very little with the three treat-

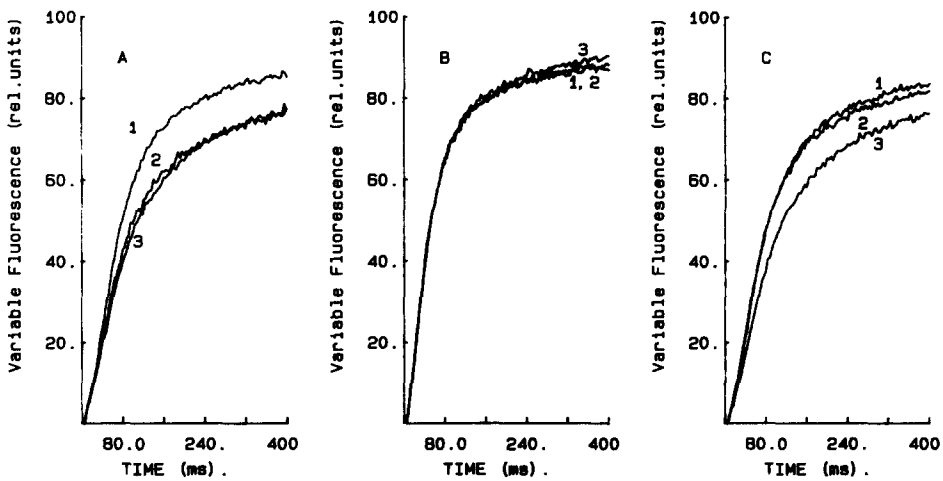

Fig. 5. Variable fluorescence versus time for thylakoids inhibited with 50 μM DCMU. The variable fluorescence for each experiment has been normalised against its maximum value. The light intensity was provided by four light-emitting diodes directed against the four sides of the cuvette containing the thylakoids. Mean light intensity on each side was 10 μmol $m^{-2} s^{-1}$. Chlorophyll concentration = 8 μg ml^{-1}. Other conditions as described in Section 2. In (A) the thylakoids were dialysed for 40 min in the dark against either a chloride (curve 1), sulphate (curve 2), or nitrate (curve 3) medium at pH 7.9. In (B), similar experiments were performed at pH 6.5 but the three curves are essentially indistinguishable and cannot be separately labelled. In (C), the pH was 7.9 but the thylakoids were dialysed against a chloride medium (curve 1), a sulphate medium in which 10 mM of sodium sulphate was replaced with 10 mM sodium chloride (curve 2), or a nitrate medium in which 10 mM sodium nitrate was replaced by 10 mM sodium chloride (curve 3).

Table 1
Parameters describing the fluorescence induction curves of broken chloroplasts dialysed against a chloride, sulphate or nitrate medium and inhibited with DCMU; all values are means ± S.D. ($n = 6$)

(A)

Anion	Phase areas (relative units)			
	α	β	Γ	δ
Chloride	120 ± 30	257 ± 36	450 ± 40	1400 ± 160
Sulphate	65 ± 22	255 ± 24	530 ± 50	3200 ± 300
Nitrate	73 ± 9	335 ± 75	700 ± 90	2500 ± 300

(B)

Anion	Rate constants ($*1000\ s^{-1}$)			
	α	β	Γ	δ
Chloride	93 ± 10	35 ± 5	5.8 ± 0.4	0.9 ± 0.01
Sulphate	98 ± 8	36 ± 4	6.8 ± 0.3	0.9 ± 0.01
Nitrate	99 ± 9	29 ± 2	5.7 ± 0.2	0.9 ± 0.01

(C)

Anion	F_0 (relative units)	F_v (relative units)
Chloride	10.7 ± 1	22 ± 2
Sulphate	10.5 ± 1.1	22 ± 2
Nitrate	10.8 ± 0.6	21 ± 1.6

ments (Table 1B) while no significant changes in the values of F_0 and F_v could be detected (Table 1C).

Repeating these experiments at pH 6.5 did not reveal any significant differences in the rise of variable fluorescence between thylakoids which had been dialysed against the three media, as shown in Fig. 5B where the chloride curve lies between the other two curves and cannot be distinguished.

When 10 mM NaCl replaced an equivalent amount of sodium sulphate in a sulphate medium at pH 7.9, the slow rise of F_v was completely abolished (Fig. 5C, curve 2) but a similar addition of sodium chloride to a nitrate medium produced an induction curve (Fig. 5C, curve 3) which was clearly slower than the chloride control (curve 1). In a parallel study with the modulated oxygen electrode, thylakoids were initially bathed in a chloride medium. At the start of a 30-min dark period, the bathing medium was changed to a sulphate medium or a sulphate medium containing 10 mM sodium chloride. There was an inhibition of 42% in the sulphate medium but 22% in the sulphate medium containing the sodium chloride, and thus the inclusion of the the chloride did not abolish the inhibition of oxygen evolution.

4. Discussion

It was shown by Theg and Homann [1] that pea thylakoids isolated in a chloride-free sulphate medium will manifest a significant chloride effect if the pH is sufficiently alkaline (e.g., 8.0) or if the thylakoids have been exposed to an uncoupler in the dark, in which case the pH dependence of the chloride effect is largely abolished. It was proposed that there are within thylakoid membranes regions in which protons are sequestered, and the presence of these protons prevents the loss of chloride ions from the membranes [1]. The protons are sustained in the membrane by light-driven electron transport but leak away slowly in the dark in an alkaline medium [1]. At low pH values the protons do not escape easily and so it is necessary to change the membrane permeability with an uncoupler to produce a chloride effect [1]. This model can account for the results found here with sulphate media, where the inhibition produced at pH 7.9 was enhanced by darkness, and there was no inhibition at pH 6.5 (Figs. 2 and 3). However, the findings with the nitrate medium do not seem to require the release of sequestered protons, since there is no increased inhibition found if the nitrate medium is introduced in the dark (Fig. 1) and the inhibition occurs as readily at pH 6.5 as pH 7.9 (Fig. 3). It is concluded that Theg and Homann's model does not describe the mechanism involved in replacing chloride with nitrate in the thylakoid membrane.

The sequestered proton model [1] did not specifically involve structural changes in Photosystem II, but these were considered in detail by Wydrzynski et al. [12]. It was found that Photosystem II membranes incubated in 20 mM MES buffer (pH 6.3) took more than 10 h to exhibit the maximal stimulation of electron transport by chloride and showed an increasing irreversible loss of activity [12]. These changes were speeded up by incubation at pH 8.0 or in the presence of sulphate [12]. They suggested that permanent structural changes to Photosystem II might have to occur before electron transport becomes sensitive to the presence of chloride in the bathing medium [12]. The results seen here with sulphate do not conflict with this suggestion in that the inhibition of oxygen evolution was detected at pH 7.9 but not at 6.5. However, the inhibition of oxygen evolution with nitrate occurred just as rapidly at pH 6.5 as 7.9 and the extent of the inhibition was very similar, which is not consistent with the pH dependence of the structural changes suggested to produce the chloride effect in [12]. Also, nitrate-induced inhibition of oxygen evolution was to a large extent reversible and the phase angle was completely reversible. The latter indicates that k_{et} had regained the value it had prior to chloride removal and any active Photosystem II complexes appeared to be functioning normally. Thus these observations with nitrate media are inconsistent with the hypothesis that Photosystem II has to undergo an irreversible structural change before a chloride effect is manifested.

The removal of chloride from the thylakoid bathing medium at pH 7.9 slowed the rise of variable fluores-

cence (Fig. 5A) and thus slowed the closure of Photosystem II reaction centres [25]. This phenomenon is not a necessary part of the inhibition of oxygen evolution, since it does not occur under conditions where the latter effect does, i.e., at pH 6.5 with a nitrate medium (Fig. 5B) and at pH 7.9 with a sulphate medium containing 10 mM sodium chloride (Fig. 5C). It is possible that the slow rise of fluorescence arises from changes in Photosystem II which are distinct from those causing the inhibition of oxygen evolution. No changes in the values of F_o and F_v were detected in these fluorescence observations, but there were large changes in the phases of the complementary area in the absence of chloride (Table 1A). The existence of such phases has been explained in terms of different antenna sizes and also different degrees of connectivity, so that the α phase exhibiting non-exponential kinetics is thought to be due to photosynthetic units which can exchange excitation energy while photosynthetic units which cannot exchange excitation energy exhibit exponential kinetics [26,27]. These ideas were developed in some detail by the work of Butler and colleagues (for a review, see [28]). A more recent theoretical analysis has shown the importance of the rate constants controlling the formation and disappearance of the radical pair on the fluorescence induction curve [29]. Whatever the precise combination of factors which produced the observed fluorescence effects, it appears likely they involve either changes in the chlorophyll-binding proteins or radical pair formation at P680, neither of which has been thought previously to be a chloride-sensitive process. These fluorescence changes could be evidence of a different chloride-binding site from the site which influences electron transport, but it is also possible that both electron transport and variable fluorescence are controlled by the same chloride-binding site via structural effects which are modulated by the experimental conditions such as pH.

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