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LIGHT-, pH- AND UNCOUPLER-DEPENDENT ASSOCIATION OF CHLORIDE WITH CHLOROPLAST THYLAKOIDS

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Studies of the association of Cl^- with Photosystem (PS) II in CF_1-containing thylakoid membranes revealed that photosynthetically active Cl^- is retained in a Cl^--free medium unless it is sufficiently alkaline, uncoupling conditions are established and light is excluded. After treatment under such conditions, electron transport from water became dependent on added Cl^- under all conditions. Quantitative measurements of $^{36}Cl^-$ retention in the light revealed that there were about five Cl^- anions present in Cl^--sufficient chloroplasts per PS II reaction center, and one-fourth of that in Cl^--deficient samples. Uncouplers representing three different types of uncoupling mechanism were found to be effective mediators of Cl^- release from thylakoids. Since the ability to collapse a proton gradient probably is the only property shared by all the tested uncouplers, a proton gradient may be involved in the retention of Cl^-. As uncoupler-mediated Cl^- release did not depend on preillumination of our samples, a long-lived proton gradient must exist in dark-adapted chloroplasts which may not span the whole thickness of the thylakoid membrane. It is postulated that the Cl^- active in PS II reactions resides in a special membrane domain from which protons slowly equilibrate with those in the bulk solutions. Cl^- is thought to be released to the bulk phases only when the pH of the membrane domain is raised above a certain threshold by the action of uncouplers. This domain may be identical to the intramembranous compartment which has been postulated to be associated with PS II (Prochaska, L.J. and Dilley, R.A., (1978) Front. Biol. Res. Energ. 1, 265–274).

Introduction

In 1944, Warburg and Lüttgens [1] reported that the presence of Cl^- is essential for maximal rates of electron transport in chloroplasts. During the next 20 years it was observed that a Cl^- dependence of electron transport occurred only at alkaline pH [2], and that cyclic electron flow around PS I was immune to Cl^- deficiency [3]. In a series of papers in 1969, Hind and his collaborators [4–7] further investigated the photosynthetic Cl^- requirement. As a measure of the dependence of electron transport on added Cl^-, they introduced the term 'Cl^- effect', which is defined as the rate of electron transport in Cl^--free chloroplasts after the readdition of Cl^- (5–10 mM) divided by the rate obtained without Cl^- addition. They found that a Cl^- dependence of electron flow was often demonstrable only after the chloroplasts had been heated and/or exposed to alkaline pH for a brief period, that basal electron transport was independent of Cl^- availability in the

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Tricine, *N*-tris(hydroxymethyl)methylglycine; PS, photosystem; CF_1, hydrophilic component of the chloroplast coupling factor; Chl, chlorophyll.

assay medium, and that the pH dependence of the Cl^- effect could be abolished by uncoupling with EDTA washing during the chloroplast isolation procedure [8]. They were also able to identify unequivocally the oxidizing side of PS II as the site of Cl^- action.

The latter finding suggested that Cl^- is a cofactor in the photosynthetic water-splitting reaction, and that it interacts with the putative manganese-containing water-photooxidase [9]. Kelly and Izawa [10,11] gave additional support to this contention by demonstrating that photosynthetically active manganese is more easily extracted in the absence of Cl^- by such treatments as Tris and hydroxylamine washing [9]. Quite recently, Muallem and Izawa [12] and Muallem et al. [13] obtained evidence which suggested that Cl^- depletion alters the lifetimes of the S_2 and S_3 states of the manganese-containing enzyme [14].

The experiments reported in this paper were destined to explain three peculiar observations concerning the Cl^- effect which are difficult to reconcile with the notion that Cl^- is a cofactor of the water-splitting enzyme. First, the induction procedure which Hind et al. [4] found necessary to produce Cl^--depleted chloroplasts seemed, according to their own measurements, to remove micromolar amounts of Cl^- from the thylakoids, while an addition of millimolar amounts was required to achieve significant regeneration of Hill activity. Indeed, the necessity of a special procedure for inducing a Cl^- effect is puzzling, since thylakoid membranes are quite permeable to Cl^- [15] and should become Cl^- deficient simply by washing in Cl^--free media. Second, the magnitude of the Cl^- effect reported by Hind et al. [4] changed from approx. 1.0 in coupled chloroplasts to more than 2.0 in uncoupled chloroplasts, not so much because of the well known stimulation of electron transport in the presence of uncouplers, but rather because the uncoupler seemingly inhibited the *minus* Cl^- rate. Finally, it is difficult to understand why Cl^- would be required for oxygen evolution below pH 7.6 only in those chloroplasts from which the coupling factor had been removed during their isolation.

Many of our experiments are concerned with the mechanism by which Cl^- is released into Cl^--free media from its site in PS II. Usually, the transition from the Cl^--sufficient to a Cl^--deficient state was inferred from a decrease in the rate of oxygen-evolving electron transport, not from direct measurements of chloroplast Cl^- concentrations (but see Table I). Whenever tested, however, this loss of Hill activity did not occur in media containing Cl^-, and was largely, though not completely [4], reversible by addition of Cl^-. We therefore believe that the assumed correlation between electron transport and Cl^- concentration in PS II is justified.

Materials and Methods

The chloroplasts used in most of our experiments (hereafter called stock chloroplasts) were isolated at 4°C from pea seedlings (*Pisum sativum*, var. Progress 9) by grinding briefly in a Waring blender in a Cl^--free medium containing 200 mM sucrose, 25 mM Na-Hepes, pH 7.5, and 5 mM $MgSO_4$ (grinding buffer). The slurry was passed through four layers of Miracloth, and the heavy particles were collected by centrifugation at $2000 \times g$ for 10 min. The pellet was washed twice in an identical medium, and then suspended in a medium in which the Hepes was replaced by 25 mM Na-Pipes at pH 7.0 (suspension buffer). The suspension was centrifuged at $2000 \times g$ for approx. 10 s, after which the supernatant was poured off and kept on ice in dim light until use. Occasionally, stock chloroplasts were incubated in the presence of an uncoupling concentration of $(NH_4)_2SO_4$ at room temperature in high pH media, then washed free of $(NH_4)_2SO_4$ and resuspended in ice-cold suspension buffer (uncoupler-treated chloroplasts). The concentrations of chorophyll and $(NH_4)_2SO_4$ during the incubation period and the number of subsequent uncoupler-free washes are noted in the figure and table legends. To prepare the NH_2OH-treated chloroplasts used in the experiment of Table I, stock chloroplasts were incubated for 20 min in the presence of 4 mM NH_2OH, then washed once and resuspended in suspension buffer.

Rates of $K_3Fe(CN)_6$ photoreduction were measured spectrophotometrically at 420 nm in stirred media as described in Ref. 16. Unless otherwise indicated, the assay medium contained 40 mM sucrose, 25 mM Na-Tricine, pH 8.1, 5 mM $MgSO_4$ and 0.6 mM $Fe(CN)_6^{3-}$ in a total volume of 2.5 ml; other additions are noted in the legends. Chlorophyll was estimated by the method of MacKinney [17] and light inten-

TABLE I

Cl^- RETENTION BY THYLAKOID MEMBRANES

Reaction sequence: t = 0; 100 µl of chloroplasts at 1 mg/ml were placed into a test tube. To allow the release of endogenous $^{35}Cl^-$, 0.1 mM $(NH_4)_2SO_4$ and 15µl of 50 mM NaOH (the amount required to raise the pH from 7.0 to 8.0) were added. t = 2 min; approx. 10 mM $Na^{36}Cl$ added. t = 4 min; chloroplasts diluted in the light or the dark with 5 ml assay medium containing 1 mM $(NH_4)_2SO_4$ and where indicated, 5 µM DCMU. t = 5 min; suspension passed through a Millipore filter apparatus under intense white light (600 W/cm^2) was washed twice with 4 ml assay medium (minus $Fe(CN)_6^{-3}$), plus 25 mM $Na^{35}Cl$. Procedures for NH_2OH treatment and determination of the radioactivity remaining with chloroplasts on the filters are described in Materials and Methods.

Conditions during 1 min test period	Conditions during filtering period	nmol $^{36}Cl^-$ retained	nmol $^{36}Cl^-$/mg Chl	$^{36}Cl^-$/300 Chl
Red light	white light	1.49	15.2	4.6
Dark	white light	0.43	4.4	1.3
Red light + DCMU	white light	0.73	7.4	2.3
Red light with NH_2OH-washed chloroplasts	white light	0.77	7.9	2.4

sities were determined with an ISCO Model SR spectroradiometer.

Two basic experimental procedures were used and are briefly outlined below. In the first, chloroplasts were added to a cuvette that had been prefilled with assay medium and the indicated compounds, incubated in the dark for a specified period of time, usually 2 min, and then the rate of $Fe(CN)_6^{3-}$ reduction was recorded. In the second procedure, termed a dilution experiment, a small volume of the chloroplast suspension, say 60 µl, was added to an empty cuvette, followed by the addition of a few microliters of a Cl^- solution to a concentration of approx. 2.5 mM. After 1 or 2 min, 2.5 ml of assay medium were added to the cuvette, diluting the Cl^- concentration to about 60 µM, and the rate of electron transport was recorded. In some experiments, no Cl^- was added to the small volume of chloroplasts before dilution.

The amount of $^{36}Cl^-$ which remained with the chloroplasts on Millipore filters (Table I) was determined by placing the filters into scintillation vials and decolorizing them by a 4 h incubation at 60°C following the addition of 1.0 ml of 30% H_2O_2. The vials were cooled for 30 min at 4°C, then 5 ml of liquid scintillation fluid were added. After quenching chemiluminescence overnight in the dark, the vials were counted with a 2 min program.

The chemicals used in this study were not contaminated with significant amounts of Cl^-, with the exceptions of Tricine and Pipes, which were recrystallized before use. Stock solutions contained Cl^- at concentrations lower than 0.1 µM, as determined by $AgNO_3$ precipitation, and we found that a combination pH electrode could be used without problems if reasonable precautions were taken (see Ref. 10). $Na^{36}Cl$ was purchased from ICN Pharmaceuticals, Inc., and the nigericin analogue X464 was donated generously by Dr. J. Berger of Hoffmann-La Roche, Inc.

Results

Retention of Cl^- by chloroplasts during their isolation in Cl^--free media and during illumination

In our search for an explanation of the strange dependence of the Cl^- effect on uncoupling conditions and pH, we noticed that the rate of the Hill reaction in Cl^--free media depended on the time at which an uncoupler was added. As seen in Fig. 1, when stock chloroplasts were incubated in assay medium with X464 for 2 min prior to illumination, the *minus* Cl^- rate was low (trace b). In contrast, the rate was much higher when X464 was added in the light (trace d). We also discovered that after incubation with 2.5 mM Cl^-, stock chloroplasts could sustain high rates of electron transfort for 3 or 4 min following a 40-fold dilution in the light. When the dilution step was separated from the measurement of electron

transport by an intervening dark period, however, the Hill activity was found to be low. On the basis of these observations we developed a working hypothesis according to which stock chloroplasts retain significant amounts of Cl^- in PS II during their isolation in Cl^--free media, and that this essential Cl^- equilibrates with the bulk solution only in the presence of uncouplers and in darkness.

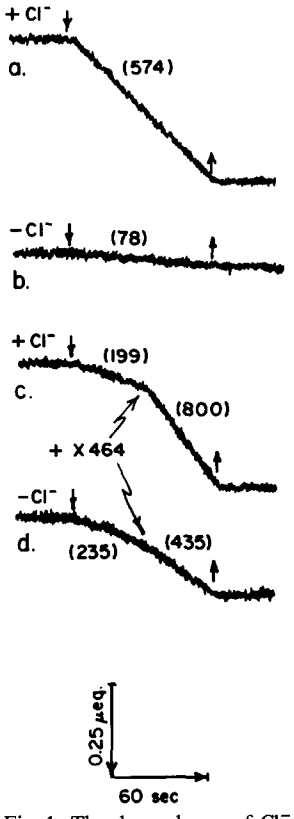

Fig. 1. The dependence of Cl^--free electron transport on the time of addition of an uncoupler. Stock chloroplasts at 12 μg Chl/ml incubated in a prefilled cuvette for 120 s in either the presence (traces a and b) or absence (traces c and d) of X464. Reaction conditions: assay medium (40 mM sucrose, 25 mM Na-Tricine, pH 8.1, 5 mM $MgSO_4$ and 0.6 mM $Fe(CN)_6^{3-}$) and where present, 10 mM NaCl and 1 μg/ml X464. Numbers in parentheses correspond to rates of electron transport in μequiv./mg Chl per h; downward and upward arrows indicate light on and off, respectively.

In agreement with such a hypothesis, we were able to assess the amount of Cl^- retained by stock chloroplasts at its site in PS II by comparing their Hill activity after dilution in the light and after pretreatment with uncouplers in the dark and subsequent washing. As seen in Fig. 2, stock chloroplasts appeared to be fully Cl^- sufficient when diluted in the light without prior addition of Cl^- (traces a and b). In contrast, uncoupler-treated chloroplasts reduced $Fe(CN)_6^{3-}$ at low rates when Cl^- was withheld from the suspension medium before dilution (traces c and d).

Direct confirmation of the retention of added Cl^- by thylakoids during a dilution experiment was achieved through the use of the $^{36}Cl^-$ isotope. As seen in Table I, when stock chloroplasts were diluted 40-fold in the light they retained approx. 3.5-times as much $^{36}Cl^-$ as those diluted in darkness. DCMU-poisoned and NH_2OH-treated chloroplasts retained about half as much $^{36}Cl^-$ following a dilution in the light as did the uninhibited sample. These data show

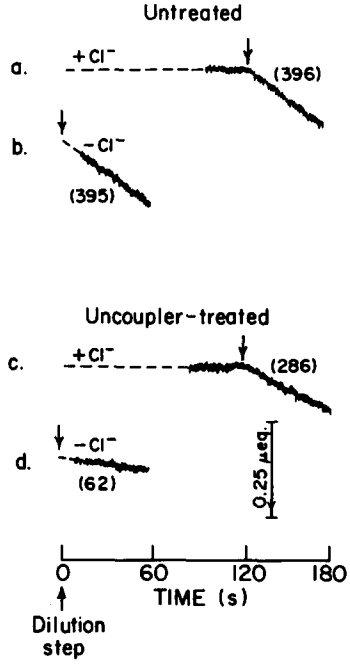

Fig. 2. Effect of uncoupler treatment on Hill activity in a dilution experiment performed in the light without prior addition of Cl^-. Traces a and c: chloroplasts dark incubated for 120 s in prefilled cuvette in the presence of 10 mM NaCl. Traces b and d: chloroplasts diluted in the light without prior addition of Cl^-. Reaction conditions: stock (a and b) or uncoupler-treated (c and d) chloroplasts at 12 μg Chl/ml, assay medium and 0.5 mM $(NH_4)_2SO_4$. Preparation of uncoupler-treated chloroplasts: 67 μg Chl/ml and 16.7 mM $(NH_4)_2SO_4$; three washes without $(NH_4)_2SO_4$.

that the increased potential of illuminated chloroplasts for Cl^- retention was a consequence of electron transport. Assuming 300 Chl/PS II unit [18], and no significant $^{36}Cl^-/^{35}Cl^-$ exchange during washing, the results also suggest that, in the Cl^--sufficient state, about five Cl^- are present per PS II trap.

The role of uncouplers in the release of Cl^-

The experiments reported in the previous section demonstrated that stock chloroplasts retain functional Cl^- unless they are incubated in the dark. Furthermore, the dependence of the rates of electron transport obtained in Cl^--free media on the time of uncoupler addition had suggested that Cl^- could be released from nonilluminated thylakoids only in the presence of an uncoupler during darkness. A simple way to verify the requirement of both an uncoupler and darkness is shown in Fig. 3. It can be seen that a decline in Hill activity indicative of Cl^- release in a dilution experiment was noticeable only when a dark period followed the addition of the uncoupler, $(NH_4)_2SO_4$. The experiment of Fig. 4 demonstrates that the uncoupler-mediated decrease in Hill activity in Cl^--free media required a 10-fold lower concentration of X464 than was necessary to uncouple fully electron from photophosphorylation.

We were puzzled by the fact that the uncoupler effect we had observed occurred in the dark and did not depend in any way on preillumination of the samples. Such dark-adapted chloroplasts are not expected to have a proton gradient which the uncouplers could disturb. In seeking to determine wether these agents facilitated Cl^- release by some other mechanism, we compared representatives of three types of uncouplers which are known to differ in their mechanisms of pH equilibration [19]. Since the ability of these compounds to collapse a proton gradient was probably their only common property, a similar effectiveness in creating Cl^- deficiency

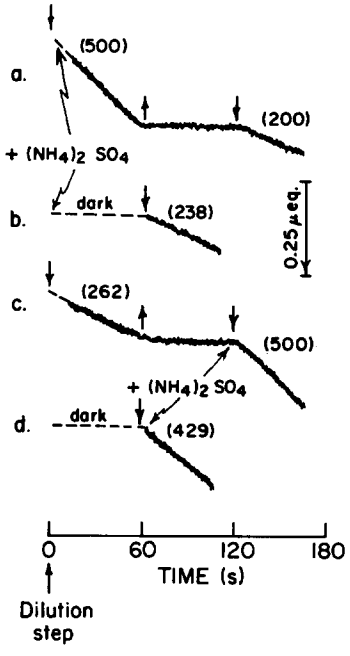

Fig. 3. The dependence of Cl^--free electron transport on light and uncoupling conditions. Traces a and c: stock chloroplasts with 2.5 mM Cl^- diluted in the light with assay medium, $(NH_4)_2SO_4$ added at $t = 0$ and 120 s, respectively. Traces b and d: chloroplasts diluted in the dark, $(NH_4)_2SO_4$ added at $t = 0$ and 60 s, respectively. Reaction conditions as in Fig. 2.

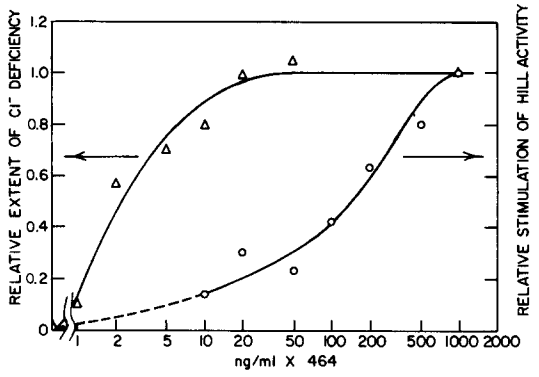

Fig. 4. Dependence of Cl^--sufficient electron transport, and of the decline of Hill activity in Cl^--free media during darkness, on uncoupler concentration. (○) Stock chloroplasts (14 μg Chl/ml) dark incubated for 120 s in a cuvette prefilled with assay medium, 10 mM NaCl and the specified amount of X464. 100% stimulation corresponds to the difference between rates recorded in the absence of X464 and with 1 μg/ml X 464. (△) Stock chloroplasts made to 2 mM Cl^- and diluted 48-fold in the light with assay medium 60 s later to 14 μg Chl/ml. The light was immediately extinguished and various amounts of X464 were added. After 60 s darkness, the light was turned on, X464 was brought to a concentration of at least 1 μg/ml, and the rate of electron transport was recorded. The difference between Hill activities remaining after dark incubation without X464 and with 1 μg/ml X464 was set equal to 100%. Each point is an average of two determinations.

would indicate the presence of a long-lived dark proton gradient. Consistent with this notion, the results presented in Table II show that all the uncouplers tested caused a decrease in Hill activity in Cl^--free media during an incubation in the dark. The behavior of tripropyltin, which mediates a Cl^-/OH^- exchange across the thylakoid membrane [20], is noteworthy. This compound is known to act as a Cl^--dependent uncoupler in chloroplasts [21,22], but can also inhibit basal electron transport under certain circumstances [21]. The latter effect may explain the low rate listed in Table II obtained during the first light period before X464 was added. Nevertheless, since we suspected that the activity of OH immediately after chloroplast dilution would be lower at the Cl^- site than in the bulk phases (see below), we were surprised that tripropyltin apparently did not cause Cl^- to be released. It is possible that tripropyltin was physically excluded from the Cl^- site, or alternatively, that the rate of Cl^-/OH^- exchange was too slow to be detected by our experiments.

At first, our results with valinomycin seemed inconsistent with the hypothesis that uncoupling conditions are required for the release of Cl^- from thylakoids. However, valinomycin has been shown to be a weak uncoupler in chloroplasts [23], and recently Ho et al. [24] have demonstrated that less tha 10 μM valinomycin increases the rate of efflux of protons from preilluminated thylakoids in the dark. Considering the fact that very low amounts of uncouplers are needed for Cl^- release, the behavior of valinomycin in our experiments may not be surprising.

The discovery that uncouplers are required for Cl^- release explains why Hind et al. [4] found basal electron flow to be independent of added Cl^-. Our experiments suggested that Cl^--dependent coupled electron transport would be observed after an uncoupler had been added to chloroplaststst suspended in Cl^--free assay media and then removed a few minutes later. The results of such an experiment are given in Fig. 5. Traces a and b were recorded with stock chloroplasts as in traces c and d of Fig. 1, and traces c and d were obtained with chloroplasts which had been uncoupled with 1 mM $(NH_4)_2SO_4$ before being washed by two centrifugations. Comparison of the final *plus* Cl^- rates (a and c) indicates that the incubation with $(NH_4)_2SO_4$ was slightly inhibitory. This complication did not, however, prevent an unambiguous interpreta-

TABLE II

THE EFFECT OF MEMBRANE-ACTIVE AGENTS ON DECLINE OF HILL ACTIVITY IN Cl^--FREE MEDIA DURING DARKNESS

Reaction conditions: assay medium and 12 μg Chl/ml. Stock chloroplasts at 2.5 mM NaCl were diluted in the light at $t = 2$, and the indicated compounds added immediately thereafter. Light off and on again at $t = 60$ and 120 s, respectively. Electron-transport rates (μequiv./mg Chl per h) measured during the two illumination periods. If the test compound was not an uncoupler, 1 μg/ml X464 was added at $t = 120$ s.

Compounds added with dilution	Rate during 1st light ($t \leq 60$ s)	1 μg/ml X464 added before 2nd light?	Rate during 2nd light ($t \geq 120$ s)	1st/2nd
Expt. 1				
None	159	yes	474	3.0
1 μg/ml X464	357	no	129	0.36
1 μM FCCP	312	no	75	0.24
2.6 μM gramicidin D	411	no	69	0.17
1 mM $(NH_4)_2SO_4$	204	no	149	0.73
Expt. 2				
1 mM $(NH_4)_2SO_4$	588	no	155	0.26
20 μM A23187	465	no	16	0.03
20 μl/ml ethanol	299	yes	480	1.6
50 μM tripropyltin	40	yes	490	12.3
5 μM valinomycin	75	yes	64	0.86
5 μM valinomycin + 10 mM NaCl	57	yes	373	6.5

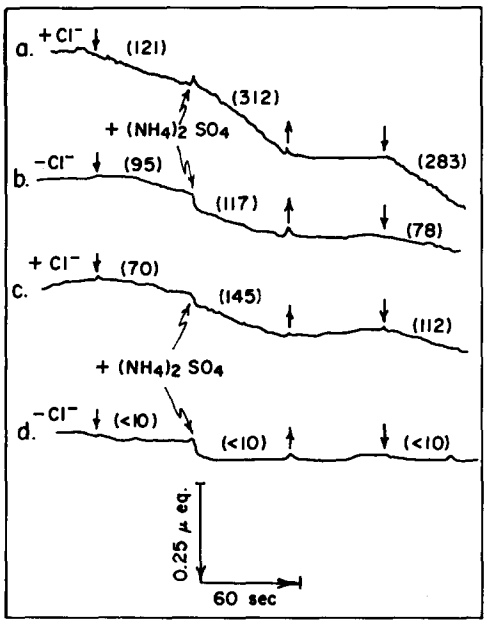

Fig. 5. Cl^- dependence of basal electron transport. Traces a and b (stock chloroplasts) and c and d (uncoupler-treated chloroplasts: stock chloroplasts incubated for 120 s in 1.5 ml assay medium without $Fe(CN)_6^{3-}$ plus $(NH_4)_2SO_4$, centrifuged and washed once in same solution lacking $(NH_4)_2SO_4$, then resuspended in 2.5 ml assay medium). Reaction conditions: assay medium, 2 mM $(NH_4)_2SO_4$, 16.4 μg Chl/ml and 10 mM NaCl (when present). Recordings obtained as in Fig. 1, traces c and d. Other details as in Fig. 1.

tion of the results. While basal electron transport of coupled stock chloroplasts displayed a Cl^- effect of only 1.3, recoupled chloroplasts showed a Cl^- effect of more than 7.

The kinetics of Cl^- release

The kinetics of the decline of Hill activity caused by the presence of combinations of uncouplers during incubation in Cl^--free media in darkness are shown in Fig. 6. Chloroplast suspensions were made to 2.5 mM NaCl, and then diluted in the dark with assay medium containing the compounds indicated. After the specified dark time, the actinic light was turned on and

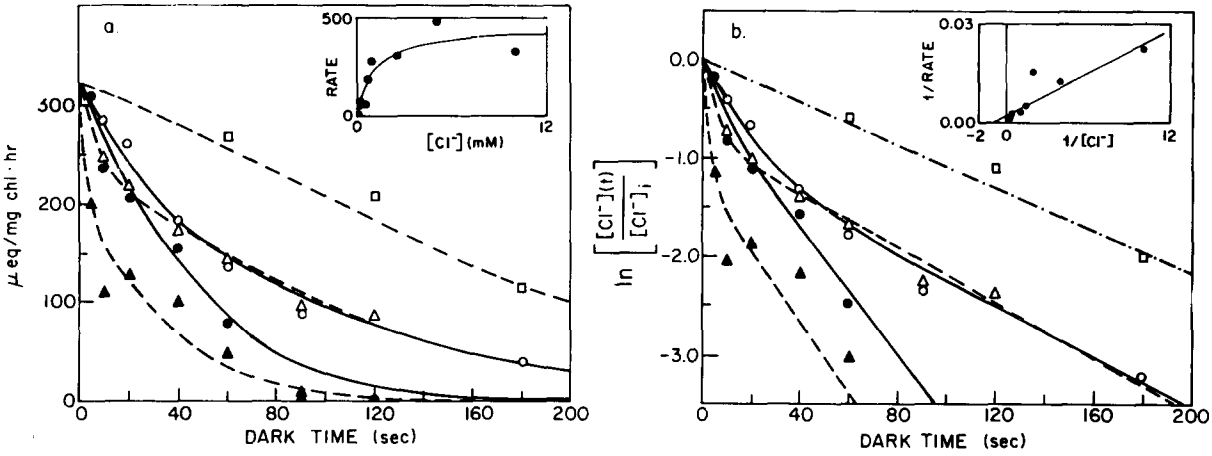

Fig. 6. The effect of ionophores on the kinetics of the decline of Hill activity in Cl^--free media during darkness. (a) Hill activity in the presence and absence of ionophores as a function of dark time. (b) First-order kinetic plots of extrapolated Cl^- release in the dark, no addition to dilution medium, but X464 added just after light on ($A_2 = 0.976, k_2 = 0.011\ s^{-1}$); ○, + X464 ($A_2 = 0.39, k_1 = 0.062\ s^{-1}, k_2 = 0.013\ s^{-1}$); ●, + X464 and tripropyltin ($A_2 = 0.64, k_1 = 0.15\ s^{-1}, k_2 = 0.032\ s^{-1}$); △, + FCCP ($A_2 = 0.45, k_1 = 0.20\ s^{-1}, k_2 = 0.014\ s^{-1}$); ▲, + FCCP and tripropyltin ($A_2 = 0.29, k_1 = 0.47\ s^{-1}, k_2 = 0.036\ s^{-1}$). (a) Inset: dependence of Hill reaction on the concentration of added Cl^-1. (b) Inset: Lineweaver-Burk plot of the data of inset a. Reaction conditions: assay medium, 12 μg Chl/ml and when added, 1 μg/ml X464, 0.47 μM FCCP and 28 μM tripropyltin. Stock chloroplasts with 2.5 mM NaCl diluted 80-fold in the dark; light on after dark times specified on the abscissae. Points in inset (a) determined after 2 min dark incubation of chloroplasts in the presence of Cl^- and 1 μg/ml X 464. For calculation of points in b see text. The lines in b were computer drawn from the equation in the text describing the change in functional Cl^- as the sum of two exponential processes. The lines in the insets were drawn from the Michaelis-Menten equation with $V = 455$ μequiv./mg Chl per h and $K_m = 1.0$ mM, and those in a were calculated by combining the above two equations.

the remaining potential for $Fe(CN)_6^{3-}$ reduction was recorded. As seen in Fig. 6a, the presence of X464 clearly hastened the fall in the rate of electron transport over that observed without an uncoupler (open circles and squares, respectively), and the rate of decline was further accelerated by tripropyltin (closed circles). When FCCP was substituted for X464 (open triangles), the potential for Hill activity dropped more quickly at dark times shorter than 30 s, and again, a further addition of tripropyltin led to an even faster decline.

The correlation between the kinetics of the decline in electron-transport activity and the release of Cl^- was judged from the experiment shown in the inset Fig. 6a. In it we determined the dependence of the Hill reaction in Cl^--depleted chloroplasts on the concentration of added Cl^-. The values for V and K_m obtained from the corresponding Lineweaver-Burk plot (Fig. 6b inset) were 455 μequiv./mg Chl per h and 1.0 mM (cf. Ref. 10), and the corresponding rate calculated from the Michaelis-Menten equation at zero dark time with 2.5 mM Cl^- was 325 μequiv./mh Chl per h. All subsequent rates were therefore normalized to this value.

Using these parameters, a first-order logarithmic plot of Cl^- release in the dark was calculated, which is shown in Fig. 6b. As can be seen, the kinetics of Cl^- release in the presence of uncoupler were always nonfirst order. It could, however, be described by the sum of two exponential processes, i.e., $[Cl^-]\ (t) = [Cl^-]_0 \cdot [A_1\ \exp(-k_1 t) + A_2\ \exp(-k_2 t)]$, where A_1 and A_2 are the amplitude factors corresponding to the two first-order rate constants, k_1 and k_2. A_2 and k_2 were determined from the slope and intercept of least-squares analyses of the last four or five points at long dark times, and since the initial and final concentrations of Cl^- were 2.5 and 0.06 mM, respectively, $A_1 = (1 - 0.06/2.5) - A_2$. k_1 was determined by trial and error.

The final slopes of the lines in Fig. 6b were approximately identical in the three experiments performed without tripropyltin, which suggests that the main effects of X464 and FCCP were to cause a fast release of Cl^- during the initial stages of the dark period. When tripropyltin was included in the dilution medium, the final slopes were again similar to each other, but were almost 3-times higher than those obtained in its absence. This might indicate that, once Cl^- had been released from its site in PS II, it encountered yet another barrier before reaching the bulk solution. This second obstacle apparently could be overcome by a tripropyltin-mediated Cl^-/OH^- exchange.

In addition to being an uncoupler, FCCP is known to be an 'ADRY' reagent, i.e., to cause an acceleration of the decay of the S_3 and S_2 states of the manganese-containing enzyme to S_1 [25]. It has been suggested by Fowler [26] and by Crofts and Wood [27] that Cl^- binds to the S_2 state and serves a charge-balancing role that is fulfilled for the other S states by the release of protons (Refs. 26–28; but see also Refs. 29 and 30]. Our finding that FCCP causes a faster initial Cl^- release than does X464 is in agreement with such a mechanism for Cl^- involvement in the water-splitting reaction.

The pH dependence of the Cl^- effect

One of the peculiar aspects of Cl^- deficiency was the observation of previous investigators that the pH dependence of the Cl^- effect in chloroplasts with an intact ATPase was abolished after removal of CF_1 by EDTA-washing [6,10]. The pH dependence shown

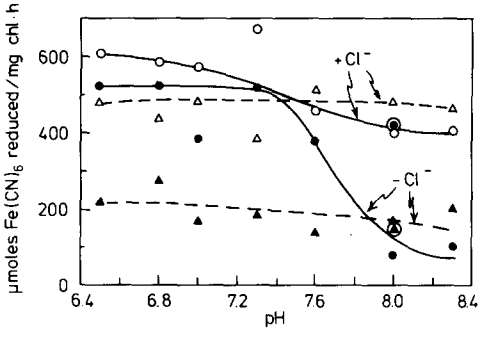

Fig. 7. Abolishment of the pH dependence of the Cl^- effect by uncoupler treatment. Chloroplasts dark incubated for 120 s in prefilled cuvette. (○) Stock chloroplasts. (△) Uncoupler-treated chloroplasts (68 μg Chl/ml and 1 mM $(NH_4)_2SO_4$; one wash without $(NH_4)_2SO_4$). Open and closed symbols correspond to *plus* and *minus* Cl^- rates, respectively. The encircled symbols represent rates measured in a light dilution experiment without prior addition of Cl^- as in Fig. 2, trace b. Reaction conditions: assay medium with buffers listed below, 12 μg Chl/ml, 4.8 μM gramicidin D and 10 mM NaCl (when present). Buffers: Na-Mes, pH 6.5; Na-Pipes, pH 6.8 and 7.0; Na-Hepes, pH 7.3 and 7.6; Na-Tricine, pH 8.0 and 8.3.

for our stock chloroplasts (with CF_1) in Fig. 7 (circles) is very similar to the plot seen in Fig. 4 of Ref. 4. Clearly, added Cl^- was not required for oxygen evolution at pH values lower than 7.6. We suspected that this apparent lack of a Cl^- requirement was due to an incomplete Cl^- removal at low pH. If so, we wondered if removal of the residual Cl^- by an incubation in uncoupler-containing alkaline media would render the Cl^- effect pH independent. The circles symbols in Fig. 7 represent the rates of electron transport obtained with untreated and uncoupler-treated chloroplasts following a dilution in the light without prior addition of Cl^-. Assuming that under such conditions the measured decline in Hill activity can be attributed to a loss of Cl^-, we used this test to ascertain that the uncoupling treatment was indeed effective in removing much the the functional Cl^-. The triangles correspond to data obtained with the uncoupler-treated chloroplasts after a 2 min incubation in reaction medium. As predicted, they displayed a Cl^- effect independent of pH.

Having shown that removal of the coupling factor is not a requirement for obtaining a pH-independent Cl^- effect, it remained to be answered why chloroplasts retained Cl^- at low pH. As an explanation, we postulated that the uncoupler-mediated Cl^- release requires a high pH of the suspension medium and is ineffective at low pH.

To test this idea, we used the following approach. It involved dilution of thylakoids in the light, followed by a pH change from a value at which uncouplers allow the release of Cl^- to one at which a significant Cl^- effect is generally not seen in CF_1-containing chloroplasts. The experiment of Fig. 8 involved a pH change from 7.8 to 6.8 brought about by an injection of a predetermined amount of H_2SO_4 into the reaction vessel at the time indicated by the broad arrows. If our postulate was correct, Cl^- release should have occurred when the pH transition followed, but not when it preceded, a dark incubation in the presence of an uncoupler. In traces a and c, the pH of the medium was high during the 60 s dark period, and so the rates measured subsequent to it were Cl^- limited. Note that the second rate in trace c was recorded at pH 6.8, again demonstrating that a Cl^- requirement at low pH can be induced in stock chloroplasts by removal of the residual Cl^-. In traces b and d, the pH was lowered before the dark period,

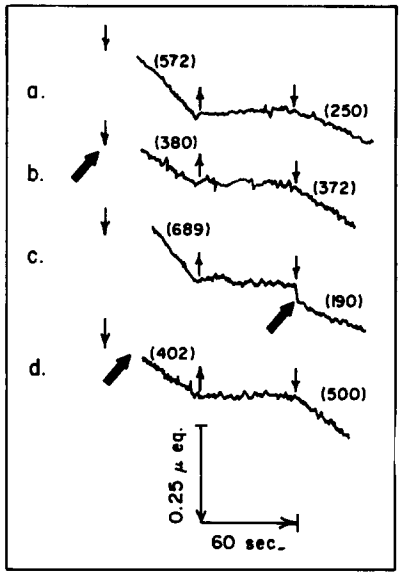

Fig. 8. The pH dependence of uncoupler-mediated decline of Hill activity in Cl^--free media during darkness. Traces: a, no pH change; b, pH of assay medium lowered before use in dilution; c, pH lowered after dark period; d, pH lowered 15 s after dilution. Stock chloroplasts with 2.0 mM NaCl kept on ice until use. At $t = 0$, 50 μl of the chloroplast suspension were placed into an empty cuvette and immediately diluted in the light with assay medium to 11.2 μg Chl/ml. 1 μg/ml X464 was added at $t = 15$ s. Broad arrows indicate the time of addition of 25 μl 0.1 N H_2SO_4, which changed the pH of the assay medium from 7.8 to 6.8. Other details as in Fig. 1.

and as predicted, no Cl^- escaped into the bulk solution during this time.

The possible involvement of Cl^- and uncouplers with an intramembranous compartment

At this point in our investigation, some striking parallels between our data and those obtained by Dilley and co-workers [31,32] had become evident. Both appeared to suggest the existence of a metastable proton gradient in dark-adapted chloroplasts that could be collapsed by uncouplers. Dilley and coworkers [31,32], building upon the ideas of Williams [33] concerning oxidative and photophosphorylation, have hypothesized that a proton-sequestering channel resides within the thylakoid membrane into which protons derived from water oxidation are deposited and through which they may pass to the coupling factor without entering the lumen. The pH of the channel is supposedly controlled indepen-

dently of the pH of the bulk phases and equilibrates readily with the latter only when uncouplers are added to the suspension medium. Light-driven water oxidation is thought to cause channel acidification. Our data would be compatible with the assumption that Cl^- is held in the same, or a similar, membrane channel, and that it is released into a Cl^--free bulk solution only when the pH of the channel rises above a certain threshold. Since the channel's protons do not readily equilibrate with protons in the bulk phase, uncouplers must be added to generate Cl^- deficiency.

To test further this hypothesis, we sought a way to equilibrate the pH by uncoupler action, and then restore the channel to its original condition through removal of the uncoupler. As a readily removable uncoupling agent, we again chosen NH_3. We predicted that equilibration of the compartment's pH with alkaline media should yield chloroplasts which would show a Cl^- effect in both the coupled and uncoupled states, even when they had been preincubated with Cl^-. However, this should be true only if the chloroplasts had been kept strictly dark after removal of the uncoupler to prevent acidification of the Cl site through electron transport. Specifically, if choroplasts treated in the above-described manner were made to 2.5 mM NaCl and then diluted in the dark, we expected that in contrast to the usual situation, the Cl^- would be able to diffuse away from its site in PS II in the absence of an uncoupler. If the same chloroplasts were exposed to light during the dilution, however, water oxidation should reacidify the membrane compartment and Cl^- would be able to escape into the bulk solution only when an uncoupler had been added.

A flow chart describing the experimental procedure together with the results of one of the three experiments performed is shown in Fig. 9. In Table III, the results of all three experiments are given as ratios of rates recorded with the different reaction sequences (see the legend of Fig. 9 for an explanation of the sequences). The first two ratios are controls and provide information concerning the success of the recoupling step and the effectiveness of $(NH_4)_2SO_4$ in causing Cl^- release in the dark.

Evaluation of these experiments in view of the results predicted by our hypothesis is achieved most readily by examining the last four ratios in Table III.

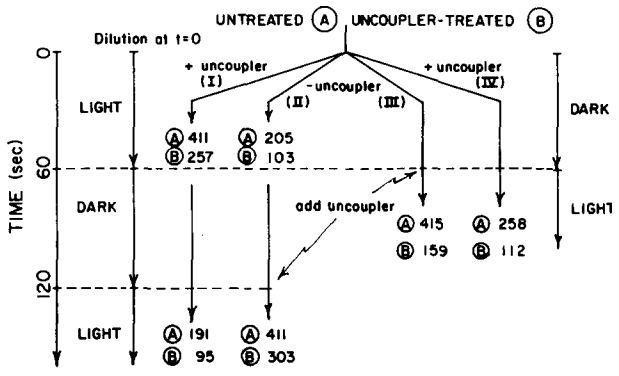

Fig. 9. The effect of prior alkalization of the Cl^- site on the uncoupler requirement for the decline of Hill activity in Cl^--free media in darkness. Flow diagram gives experimental procedure and the results of one experiment. Reaction conditions: assay medium, 12 μg Chl/ml and 1 mM $(NH_4)_2SO_4$. Roman numerals I–IV identify the reaction sequences for Table III, which were the same as those used in Fig. 3, traces a, c, d, and b, respectively, and the numbers below the arrows correspond to rates of electron transport in μequiv./mg Chl per h (average from two measurements). Untreated sample: stock chloroplasts. Uncoupler-treated sample: 560 μg Chl/ml and two uncoupling incubations performed with 1.24 and 0.25 mM $(NH_4)_2SO_4$, respectively; two uncoupler-free washes.

The ratio IV/III was expected to be 1.0 for the uncoupler-treated samples that would not, and less than 1.0 for the control samples that would require $(NH_4)_2SO_4$ for Cl^- release from chloroplasts that had not been preilluminated. The fourth ratio is a better indication of this uncoupler requirement, and should be 1.0 and less than 1.0 for the untreated and uncoupler-treated samples, respectively. II 2nd/I 1st was expected to be 1.0 for all samples, which would indicate that illumination had restored the uncoupler requirement for Cl^- release from the uncoupler-treated chloroplasts. The last ratio, III/II 2nd, indicates the extent of change in the uncoupler requirement caused by electron transport, and should be 1.0 for the control and less than 1.0 for uncoupler-treated samples. These values are all in relatively good agreement with those given in the table, and where the deviations are large, the trends in the data are always in the direction anticipated by the membrane compartment hypothesis.

It is interesting to note that although pH 8.1. media were used for all incubations with $(NH_4)_2SO_4$

TABLE III

SOME RATIOS DERIVED FROM COMBINATIONS OF RATES RECORDED AS IN FIG. 9

Expt. 1 is shown in Fig. 8; identical procedures were used in all three experiments. Uncoupler-treated chloroplasts prepared as in Fig. 2, with the following exceptions: Expt. 2, Na-Pipes, pH 7.0, was replaced by 25 mM Na-Tricine, pH 8.1, in the final resuspension medium; Expt. 3, 1440 μg Chl/ml. With reference to reaction sequences I and II, the suffixes 1st and 2nd refer to the rates recorded before and after the dark period, respectively, i.e., the ratio I 1st/II 1st for the untreated sample (A) in Expt. 1 corresponds to 411/205.

Ratio	Meaning of ratio	Expt.	Sample	
			Untreated	Uncoupler treated
I 1st/II 1st	Photosynthetic control ratio	1	2.00	2.50
		2	1.79	1.86
		3	1.91	1.79
I 2nd/II 2nd	Relative change in uncoupled Hill activity	1	0.46	0.31
	of preilluminated samples caused by dark	2	0.42	0.53
	incubation with uncoupler	3	0.30	0.36
IV/III	Relative change in uncoupled Hill activity	1	0.62	0.70
	of nonilluminated samples caused by	2	0.64	1.05
	dark incubation with uncoupler	3	0.55	0.77
III/I 1st	Relative difference in uncoupled Hill activity	1	1.01	0.62
	of nonilluminated samples after dark incubation	2	0.91	0.21
	without uncoupler	3	0.92	0.41
II 2nd/I 1st	Relative difference in uncoupled Hill activity	1	1.00	1.18
	of preilluminated samples after dark incubation	2	1.07	0.73
	without uncoupler	3	1.16	0.93
III/II 2nd	Relative change in uncoupled Hill activity	1	1.01	0.52
	remaining after dark incubation without	2	0.85	0.29
	uncoupler caused by light	3	0.79	0.44

and for the subsequent washing steps, the test sample of Expt. 2 was stored at pH 8.1 on ice until use, while those of Expts. 1 and 3 were stored at pH 7.0. The latter chloroplasts should have had a lower proton concentration inside their membrane channels than outside, which is opposite to the situation encountered in a dilution experiment with stock chloroplasts. From the fact that there were no qualitative differences between the data obtained in the three experiments, we conclude that the compartment's permeability barrier inhibits not only the efflux of protons into the bulk phases, but also their influx when the activity gradient is reversed.

Discussion

In this paper we have investigated the reasons underlying some peculiar aspects of the Cl^- requirement of chloroplasts. We found that (1) functional Cl^- is usually retained at a site in PS II during thylakoid isolation in Cl^--free media; (2) a dependence of Hill activity or added Cl^- can be obtained only after incubation at slightly alkaline pH; (3) the effectiveness of such a pH in causing Cl^- deficiency of electron transport is greatly enhanced by conditions which uncouple photophosphorylation; and (4) light-driven electron transport prevents the decline of Hill activity in Cl^--free media in the dark.

The restoration of normal Hill activity, and the prevention of its decline, by Cl^- suggested that the phenomena we observed were related to the amount of functional Cl^- at a critical site in PS II. This contention was reinforced by actual measurements of the amount of $^{36}Cl^-$ associated with thylakoids under various conditions. Therefore, our data may be used to explain a number of curious observations con-

cerning the Cl^- requirement of PS II that have been reported in the literature. Hind et al. [4], for example, found basal electron transport to be insensitive to Cl^- deficiency, and uncouplers to inhibit electron transport in Cl^--free media. Our experiments explain this by showing that chloroplasts do not in fact become Cl^- deficient until they are incubated in the presence of uncouplers (Fig. 2). Indeed, once the residual Cl^- is actually removed, coupled electron transport becomes extremely Cl^- dependent (Fig. 5). The pH dependence of the Cl^- effect in chloroplasts possessing an intact coupling factor can be similarly explained as an incomplete Cl^- removal at low pH. That EDTA-washed chloroplasts would lack this pH dependence [6,10] could also have been anticipated from our data, as the washing step which causes uncoupling is performed at alkaline pH and is followed by centrifugation in the dark. Fig. 7 shows that removal of the coupling factor is not necessary for obtaining a Cl^- effect at low pH.

Hind et al. [4] also reported that chloroplasts often did not display a significant Cl^- effect immediately after their isolation by two Cl^--free washes. This could be remedied by an induction procedure which involved transiently raising the pH of the chloroplasts suspension medium or heating the chloroplasts briefly, and the most effective induction was achieved by simultaneously heating and raising the pH. Takahama et al. [34] have shown that a temperature jump applied in the dark to preilluminated chloroplasts causes a large efflux of protons into the suspending medium. Furthermore, Baker et al. [31] and Dilley et al. [32] have used a slow temperature change to alter the degree of pH-dependent acetylation of thylakoid proteins by acetic anhydride. Since the conditions that cause lowered acetic anhydride binding and Cl^- release are apparently the same, the induction procedures of Hind et al. [4] can be viewed as a method to bring about Cl^- deficiency by increasing the proton permeability of thylakoid membranes. In our experiments, we routinely used high pH and room temperature in conjunction with uncouplers to cause Cl^- to be released from our stock samples (uncoupler-treated chloroplasts).

Our data are difficult to interpret in terms of conventional views of the organization of the thylakoid membrane [35]. That uncouplers have an effect on dark-adapted chloroplasts is remarkable, and the common influence of three different classes of uncouplers on Cl^- release (Table II) points strongly to the presence in our samples of a persistent proton gradient in the dark. The existence of such a dark gradient has been suggested implicity or explicitly by others [31, 32,34,36–38].

A small proton gradient spanning the thylakoid membrane may be present due to the action of the lumen as a Donnan phase [39]. However, since protonophoric uncouplers would not affect a ΔpH established as the consequence of a Donnan equilibrium involving nondiffusible negative charges, it cannot be the gradient responsible for our observations. Moreover, if it is true that all the buffers used by us quickly enter the lumen as reported by Junge et al. [40], any ΔpH between the inside and outside bulk phases other than the Donnan gradient should be abolished rapidly. Hence, it could be that the dark proton and Cl^- gradients in our thylakoid preparations do not span the space between the external and internal bulk solutions, but are localized in a special phase. Dilley and co-workers [31,32,41,42] have shown that the degree of reaction of iodoacetic acid and acetic anhydride with thylakoid membranes is altered by protons derived from PS II protolytic events, but not by protons originating from PS I, thus arguing for the existence of separate proton-sequestering domains for the two photosystems. As far as Cl^--binding domains are concerned, the predominating electronegative charge on the two surfaces of the thylakoid membranes [43,44] would appear to prevent extensive interactions between Cl^- and surface groups. It is possible, therefore, that there exists a compartment within the thylakoid membrane in which protons and Cl^- are held in the dark against their activity gradients. If the compartment is aqueous and the internal interfaces play a role in energy transduction, the ideas of Kell [45] on membrane proticity might be relevant to this concept.

The existence of a special compartment within the thylakoid membrane is consistent with all the data we have obtained thus far. Since the protons residing in the compartment which make up the gradient are not replenished in the dark, a small increase in the membrane's proton permeability could greatly accelerate their equilibration. One might therefore have anticipated that uncouplers would be required at low concentrations to influence sufficiently the channel pH

to cause Cl^- release (Fig. 4).

The complex kinetics of Cl^- release during a dark period (Fig. 6) could also be a consequence of Cl^- residing in a membrane channel. They indicate that the effect of uncouplers is limited to the initial periods of darkness in agreement with the uncouplers' ability to equilibrate rapidly the pH of the compartmented and bulk solutions. The accelerating effect of tripropyltin on the rate of Cl^- release is probably due to its ability to mediate a Cl^-/OH^- antiport across the thylakoid membrane [20]. Interestingly, the rate constants used in the equation from which the lines in Fig. 5a and b were calculated were both affected similarly by the addition of tripropyltin to X464 or FCCP-containing dilution media (2.45-fold increase ±3%, see the figure legend). Assuming that the rate constants reflect the speed with which Cl^- passes two distinct diffusion barriers following a dilution, their identical increase by tripropyltin suggests that both barriers are of the same nature, presumably that posed by the thylakoid membrane.

For an alternative explanation of our data, we consider that the zwitterionic buffers used by us did not readily cross the membranes of our thylakoid preparations. Hence, during the dilution step, or after an illumination, the pH of the lumen may have remained below approx. 7.6 for at least 120 s. Cl^- would not be released during this time if one assumes that it is held in PS II when the pH of the lumen is acidic or only slightly alkaline. The biphasic kinetics of Cl^- release would still suggest two diffusion barriers and, therefore, compartmentation of Cl^-. However, in the alternative concept, the compartment's pH is seen as being determined by the pH of the lumen. While all our data can presently be explained by such a model, it ought to be said that the experiments of Dilley and co-workers [31,32,41,42] on the formation of membrane protein derivatives are not so easily accommodated in it, particularly those which show a differential effect of proton gradients set up by either PS I or PS II. Experiments are presently under way to determine if there is a similar dependence of light-mediated Cl^- retention on the origin or proton gradients.

Unfortunately, our experiments do not reveal the mechanism through which Cl^- exerts its influence on the process of photosynthetic oxygen evolution. Data obtained by others indicate that Cl^- interacts with the manganese-containing enzyme [10–13] and with cytochrone *b*-559 [13,46]. Our kinetics experiment with FCCP (Fig. 6) is also consistent with an interaction of Cl^- with higher oxidation states of the manganese-containing enzyme, and the number of Cl^- retained in the light per PS II trap (Table I) was found to be approximately the same as the number of photosynthetically functional Mn atoms per trap [9].

On the other hand, more indirect effects of Cl^- on the water-splitting reaction may be suggested. Robinson and Boardman [47] postulated that Cl^- might act as a mobile, lipid-soluble anion that neutralizes protons diffusing within energy-transducing membranes en route to the ATPases, but how the effect of uncouplers observed by us could be accommodated by this model is unclear. Perhaps Cl^- might serve to counterbalance the positive charge of the protonated groups in the acidified membrane compartment such as the amines that are acylated by acetic anhydride in the experiments of Dilley and his collaborators [31,32,41,42]. Cl^- may also function to preserve the electroneutrality of the channel as protons are deposited by water oxidation and removed by the coupling factor. It is interesting to note that 15.2 nmol/mg Chl of Cl^- retained in the light (Table I) is a number remarkably similar to the 15–30 nmol/mg Chl of protons released (Dilley, personal communication) and the amount of extra acetic anhydride that can be reacted [32] as a result of adding gramicidin to chloroplasts in the dark.

While we concur with Dilley and his co-workers that a proton-sequesting compartment does seem to be present within the thylakoid membrane, we have some conceptual difficulties with one aspect of their hypothesis. It is widely believed that most of the PS II reaction centers in chloroplasts are confined to the stacked, granal regions, while the coupling factor is located primarily in the unstacked regions and on the margins of the grana [48]. In some shade plants, grana formations are extensive and can measure as much as 1 μm between margins [49]. A channel in such chloroplasts connecting a centrally located PS II trap to an ATPase on a granal margin would therefore have to span a distance of at least 0.5 μm, which seems unrealistic to us. Nevertheless, in view of the identification by Prochaska and Dilley [41,42] and Baker et al. [31] of an 8 kdalton CF_0 subunit as one of the major proteins which undergoes PS II-dependent derivative formation with acetic anhydride, we

agree with their reasoning and cannot presently offer an alternative explanation of their data.

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