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# THE ROLE OF CHLORIDE ION IN PHOTOSYSTEM II

## I. EFFECTS OF CHLORIDE ION ON PHOTOSYSTEM II ELECTRON TRANSPORT AND ON HYDROXYLAMINE INHIBITION

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## Summary

1. Chloroplasts washed with $Cl^-$-free, low-salt media (pH 8) containing EDTA, show virtually no DCMU-insensitive silicomolybdate reduction. The activity is readily restored when 10 mM $Cl^-$ is added to the reaction mixture. Very similar results were obtained with the other Photosystem II electron acceptor 2,5-dimethylquinone (with dibromothymoquinone), with the Photosystem I electron acceptor FMN, and also with ferricyanide which accepts electrons from both photosystems.

2. Strong $Cl^-$-dependence of Hill activity was observed invariably at all pH values tested (5.5—8.3) and in chloroplasts from three different plants: spinach, tobacco and corn (mesophyll).

3. In the absence of added $Cl^-$ the functionally $Cl^-$-depleted chloroplasts are able to oxidize, through Photosystem II, artificial reductants such as catechol, diphenylcarbazide, ascorbate and $H_2O_2$ at rates which are 4—12 times faster than the rate of the residual Hill reaction.

4. The $Cl^-$-concentration dependence of Hill activity with dimethylquinone as an electron acceptor is kinetically consistent with the typical enzyme activation mechanism: $E(\text{inactive}) + Cl^- \rightleftharpoons E \cdot Cl^-$ (active), and the apparent activation constant (0.9 mM at pH 7.2) is unchanged by chloroplast fragmentation.

5. The initial phase of the development of inhibition of water oxidation in $Cl^-$-depleted chloroplasts during the dark incubation with $NH_2OH$ ($\frac{1}{2}$ $H_2SO_4$) is 5 times slower when the incubation medium contains $Cl^-$ than when the medium contains $NH_2OH$ alone or $NH_2OH$ plus acetate ion. (Acetate is shown to be ineffective in stimulating $O_2$ evolution.)

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Abbreviations used: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

6. We conclude that the $Cl^-$-requiring step is one which is specifically associated with the water-splitting reaction, and suggests that $Cl^-$ probably acts as a cofactor (ligand) of the $NH_2OH$-sensitive, Mn-containing $O_2$-evolving enzyme.

## Introduction

In 1944, Warburg and Lüttgens [1] discovered that the Hill reaction in isolated chloroplasts required $Cl^-$ (Warburg's $Cl^-$ effect). Soon after the introduction of the concept of two photosystems [2,3], follow-up studies produced the first fruitful results when Bové et al. [4] demonstrated that the $Cl^-$ effect is characteristic of reactions which involve Photosystem II. Izawa et al. [5] later located the site of $Cl^-$ involvement on the oxidizing side of Photosystem II, and presented evidence that a step directly associated with the splitting of water molecules requires $Cl^-$. In view of the fact that the $Cl^-$ effect is also found in the lamellae from a green alga [6] and a blue green alga [7], it seems reasonably to assume that the $Cl^-$ requirement is a general phenomenon common to all $O_2$-producing photosynthetic organisms, even though there are some skeptics [8,9]. As yet, however, nothing is known about the mechanism of $Cl^-$ action.

During the last several years a variety of redox agents have been introduced which can interact directly with Photosystem II. A number of lipophilic oxidants have been introduced which can intercept electrons from Photosystem II [10—12]. A group of heteropoly compounds, represented by silicomolybdic acid, were shown to be capable of accepting electrons from Photosystem II in a DCMU-insensitive reaction [13—16]. In addition, the list of reducing compounds, used for electron donation to Photosystem II, has expanded considerably [17—19]. These and other recent developments in Photosystem II studies have strongly urged us to resume investigations of the $Cl^-$ effect.

In resuming the study of the $Cl^-$ effect, we have used EDTA-washed chloroplasts adopting the method described previously [5]. The reason for washing with EDTA is two-fold: (a) Functionally $Cl^-$-depleted conditions can be elicited more easily in EDTA-washed chloroplasts than in regular, buffer-washed chloroplasts [5]: (b) EDTA uncoupling releases the rate limitation imposed by the energy-coupling mechanism. This EDTA uncoupling is not only important for observing the full effect of $Cl^-$ on electron flow, but is also important in that it eliminates complications which are likely to arise from the salt effects on the rate-controlling coupling mechanism (see for example ref. 20).

## Materials and Methods

*Chloroplasts.* Three different plants served as chloroplast sources: market spinach (*Spinacia oleracea*), greenhouse-grown tobacco (*Nicotiana tabacum* var. Samson) and greenhouse-grown corn (*Zea mays*). EDTA-washed "$Cl^-$-depleted" chloroplasts were prepared essentually as described before [5], and finally suspended in a medium containing 0.2 M sucrose, 20 mM HEPES/NaOH buffer (pH 7.2) and bovine serum albumin (2 mg/ml).

*Subchloroplast particles.* The above chloroplasts were sonicated at 0—4°C

for a total of 50 s with an Artek Sonic 300 Dismembrator, operated at 80% of full power. The sonication medium was the same as the medium used for stock suspension of chloroplasts. Two centrifugal fractions were collected from the sonicate: a fraction sedimented between 3000 × *g* (6 min) and 25 000 × *g* (15 min), $F_{3-25K}$, and an ultracentrifugal fraction sedimented between 25 000 × *g* (15 min) and 140 000 × *g* (60 min), $F_{25-140K}$.

*Chemicals.* Most of the biochemicals and buffers used were from Sigma. The silicomolybdic acid and dibromothymoquinone (DBMIB) were generous gifts from Dr. A. Tsigdinos (Climax Molybdenum Co., Ann Arbor, Mich.) and Dr. N.E. Good, respectively. Chemicals used at relatively high concentrations (sucrose, buffers, etc.) were carefully tested for $Cl^-$ in $HNO_3$-acidic media using $AgNO_3$ as the precipitant (sensitivity, $10^{-5}$ M) to avoid inclusion of $Cl^-$ in our solutions. Tricine was found to contain sufficient amounts of $Cl^-$ as impurity to require it to be purified by recrystallization (cold acetone precipitation from concentrated aqueous solution). For pH adjustment of $Cl^-$-free solutions with a glass electrode, direct exposure of the solutions to the KCl/AgCl reference was avoided by using a sampling method or by use of a sodium citrate bridge. (Small volumes of solutions can easily built up a $Cl^-$ concentration of $10^{-4}$ M during a short exposure to a KCl/AgCl or KCl/calomel reference.)

*Measurements.* Reactions were measured at 22°C, in most cases using a Clark-type $O_2$ electrode. The actinic light used was a rate-saturating broad-band red light (620—700 nm, intensity, approx. 500 kergs $\cdot s^{-1} \cdot cm^{-2}$). The light source was a 500 W slide projector. When 2,6-dichlorophenolindophenol was used as the electron acceptor, the dye reduction was measured by monitoring the absorbance change of the reaction mixture at 600 nm. Reactions were run in a water-jacketed (22°C) 1-cm cell which was placed in a modified Beckman DU spectrophotometer furnished with Gilson-type electronics. A deep red light (650—700 nm; intensity 300 kergs $\cdot s^{-1} \cdot cm^{-2}$) was used as the actinic light to avoid overlapping of wavelengths with the measuring beam. The photomultiplier was protected from the actinic light by a layer of concentrated $CuSO_4$ solution and appropriate color filters. Occasionally ferricyanide reduction was measured optically (420 nm) using the same apparatus.

## Results

Before presenting experimental data, it should be made clear that experiments described in this paper were all carried out using EDTA-uncoupled, functionally $Cl^-$-depleted chloroplasts. In all experiments, with the exception of the last experiment (section 6), effects of $Cl^-$ were determined by adding $Cl^-$ (as NaCl) to the reaction mixture 2—3 min prior to illumination. The minimum dark preincubation time necessary to fully reactivate the chloroplasts by $Cl^-$ addition was about 2 min at 22°C (reaction temperature).

### *1. Effects of $Cl^-$ on DCMU-insensitive silicomolybdate reduction*

If the site of $Cl^-$ requirement is indeed at the $O_2$ evolution step of Photosystem II, then the $Cl^-$ effect should be observed invariably regardless of the site of electron acceptance. The DCMU-insensitive Hill reaction with silicomolybdate as the oxidant should therefore show a $Cl^-$ dependence in much the same

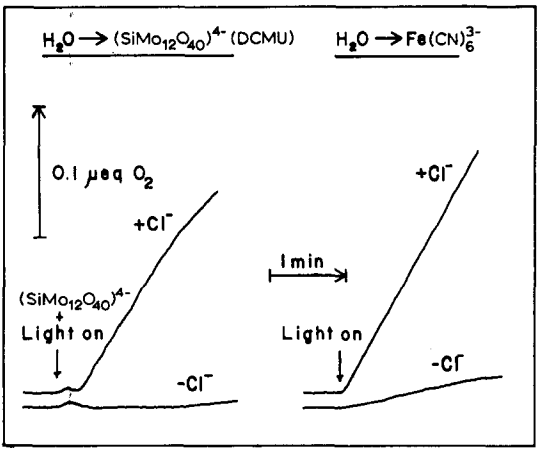


Fig. 1. Typical $O_2$ evolution traces for the Hill reaction using silicomolybdate and ferricyanide as electron acceptors in tobacco chloroplasts. The reaction mixture (1.5 ml) contained: 50 mM HEPES/NaOH buffer (pH 6.7), 10 mM NaCl (if added) and chloroplasts equivalent to 30 μg chlorophyll/ml for ferricyanide reduction and 51 μg/ml for silicomolybdate reduction. The concentration of silicomolybdate was 0.2 mM (with 5 μM DCMU) and the ferricyanide concentration was 0.25 mM.

way as does the standard, DCMU-sensitive Hill reaction. This is shown to be the case. As Fig. 1 (left, $-Cl^-$ trace) shows, in the absence of added $Cl^-$ the chloroplasts were virtually unable to transfer electrons from water to silicomolybdate. The DCMU-insensitive Hill reaction was readily restored when NaCl (10 mM) was added to the reaction mixture 2 min prior to illumination ($+Cl^-$ trace). A similarly dramatic $Cl^-$ effect was observed for the DCMU-sensitive ferricyanide Hill reaction (Fig. 1, right traces). Fig. 2 indicates that the rate of DCMU-insensitive silicomolybdate reduction saturates at about 10 mM $Cl^-$ where the

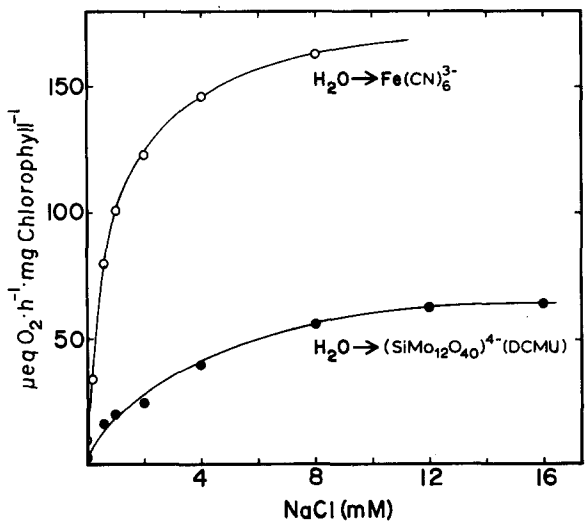


Fig. 2. $O_2$ evolution in tobacco chloroplasts as a function of $Cl^-$ concentration. The reaction mixtures used were the same as in Fig. 1 except for the varied $Cl^-$ concentrations.

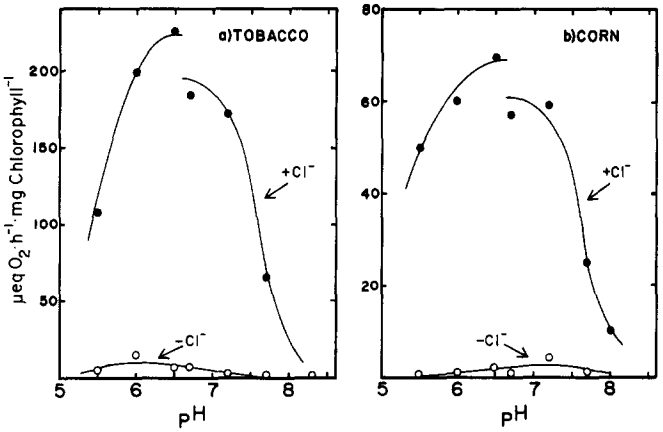


Fig. 3. pH dependence of $O_2$ production (with or without $Cl^-$) in tobacco and corn (mesophyll) chloroplasts with silicomolybdate as the electron acceptor. The reaction mixtures were the same as in Fig. 1 except for the varied pH values. Buffers used were: MES for pH 5.5—6.5. HEPES for pH 6.7—7.7 and tricine for pH 8.0 and 8.3 (all at 50 mM with NaOH). If added, $Cl^-$ (NaCl) was at 10 mM.

ferricyanide Hill reaction also saturates. Although the half-saturation concentrations of $Cl^-$ were somewhat different (0.9 mM for ferricyanide reduction and 2 mM for silicomolybdate reduction), it seems clear that the two reactions have the same $Cl^-$-sensitive step in common.

The experiments we have described so far were conducted using tobacco chloroplasts. Similar results were obtained also with corn (mesophyll) chloroplasts, except that the DCMU-resistant silicomolybdate reduction was considerably slower than in tobacco chloroplasts (data not shown).

## 2. *The effect of pH*

As shown by the pH profiles of Fig. 3, strong dependence of silicomolybdate reduction on $Cl^-$ was observed over the entire range of pH values tested (5.5—8.3). Fig. 4 shows that the DCMU-sensitive Hill reaction also sharply

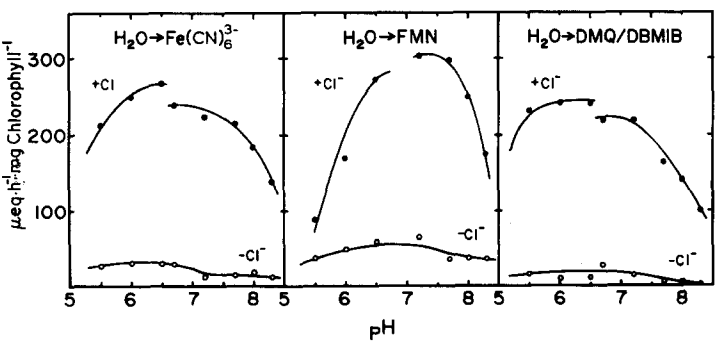


Fig. 4. pH dependence of the Hill reaction (with and without $Cl^-$) with three different electron acceptors: ferricyanide, flavin mononucleotide (FMN) and 2,5-dimethyl-*p*-benzoquinone (DMQ) with DBMIB. The ferricyanide and DMQ reactions were measured as $O_2$ evolution and the FMN reaction as $O_2$ uptake. Tobacco chloroplasts were used for the ferricyanide and FMN reactions and spinach chloroplasts for the DMQ reaction (chlorophyll, 35 μg/ml). The concentrations of electron acceptors used were: ferricyanide, 0.25 mM; FMN, 0.1 mM; DMQ, 0.5 mM (with 2 μM DBMIB). Other conditions were as in Fig. 3.

responds to $Cl^-$ at all pH values and does so regardless of the site of electron acceptance. The low-potential oxidant FMN accepts electrons strickly through Photosystem I. The reduction of 2,5-dimethyl-*p*-benzoquinone in the presence of DBMIB [21] is almost certainly a pure Photosystem II reaction [11]. In the EDTA-washed, swollen chloroplast used here, ferricyanide seemed to accept electrons in large part directly from Photosystem II (only 50% inhibition by 1 μM DBMIB).

*3. Effect of other anions*

Table I compares the effects of various anions (all added as sodium salts at 10 mM) on three reaction systems, $H_2O \rightarrow$ silicomolybdate (with DCMU), $H_2O \rightarrow$ dimethylquinone (with DBMIB) and $H_2O \rightarrow$ ferricyanide. As the table shows, the anion series determined for the activation of these three reactions were quite similar: $Cl^- > Br^- > NO_3^- \geqslant I^- \geqslant HCOO^- \geqslant HCO_3^- > F^-$ (no effect). In this series the effectiveness of the first three anions ($Cl^-$, $Br^-$, $NO_3^-$) is well known [1,4,22,23]. $I^-$ was found effective by some workers [8,22,23] but not by others [4]. This discrepancy is probably related to the potential capability of $I^-$ to donate electrons to Photosystem II [24]. The weakly effective $HCO_3^-$ has been reported as inactive [23]. Acetate was found to be totally ineffective (not shown). $F^-$ along with the di- and trivalent anions tended to give rates which were less than in the absence of added anions ("None" in the table).

*4. Effect of $Cl^-$ on Photosystem II donor reactions*

The question of whether the oxidation of exogenous reductants by Photo-

TABLE I

THE EFFECTS OF VARIOUS ANIONS ON THE RATE OF $O_2$ EVOLUTION IN $Cl^-$-DEPLETED CHLOROPLASTS WITH THREE DIFFERENT ELECTRON ACCEPTORS

Tobacco chloroplasts were used for silicomolybdate and 2,5-dimethyl-*p*-benzoquinone reduction (DMQ), and spinach chloroplasts for ferricyanide reduction. The reaction mixtures contained 50 mM HEPES/NaOH buffer (pH 7.2), 10 mM anion (indicated), and chloroplasts equivalent to 30 μg chlorophyll/ml for DMQ and ferricyanide experiments, and 50 μg/ml for silicomolybdate experiments. The concentrations of acceptors used were: silicomolybdate, 0.2 mM (with 5 μM DCMU); DMQ, 0.1 mM (with 2 μM DBMIB); ferricyanide, 0.25 mM. For other conditions, see Methods.

| Anion added * | μequiv. $O_2 \cdot h^{-1} \cdot$ mg chlorophyll$^{-1}$ | | |
|---|---|---|---|
| | Silicomolybdate (DCMU) | DMQ (DBMIB) | $Fe(CN)_6^{3-}$ |
| None | <3 (2) ** | 40 (18) ** | 33 (12) ** |
| $Cl^-$ | 122 (100) | 220 (100) | 274 (100) |
| $Br^-$ | 97 (80) | 200 (91) | 196 (72) |
| $NO_3^-$ | 28 (23) | 165 (75) | 178 (65) |
| $I^-$ | 30 (25) | 147 (67) | 86 (31) |
| $HCOO^-$ | 30 (25) | 112 (51) | 73 (27) |
| $HCO_3^-$ | 12 (10) | 85 (39) | 83 (30) |
| $F^-$ | <3 (2) | 42 (20) | 19 (7) |
| $HPO_4^{2-}$ *** | <3 (2) | 28 (12) | 22 (8) |
| $SO_4^{2-}$ *** | <3 (2) | 20 (9) | 21 (8) |

* All anions were added as sodium salts (10 mM).

** Values in parentheses are relative values.

*** Other di- and trivalent anions, such as citrate, oxalate, succinate and tartrate, all gave lower rates than in the absence of $Cl^-$ ("None" in table).

TABLE II

OXIDATION OF ARTIFICIAL REDUCTANTS VIA PHOTOSYSTEM II IN $Cl^-$-DEPLETED CHLOROPLASTS

The basic ingredients of the reaction mixtures (1.5 ml for $O_2$ assay and 2 ml for optical assay) were: 50 mM HEPES/NaOH buffer (pH 7.2), 10 mM NaCl (if added), indicated electron donors and acceptors, and chloroplasts equivalent to 33 μg/ml. The concentrations of electron donors and acceptors used were: ferricyanide, 0.25 mM; FMN, 0.1 mM; D-ascorbate, 10 mM; catechol, 0.5 mM; 1.5-diphenylcarbohydrazide (DPC), 0.5 mM; $H_2O_2$, 10 mM; 2,5-dimethyl-*p*-benzoquinone (DMQ), 0.4 mM. For other conditions, see Methods. $Cl^-$ data for the donor reactions are not included because $Cl^-$ addition activated the water oxidation and no meaningful information was obtained from the results.

| System | $Cl^-$ | μequiv. · $h^{-1}$ · mg chlorophyll$^{-1}$ | Assay |
|---|---|---|---|
| $H_2O \rightarrow Fe(CN)_6^{3-}$ | + | 348 | $O_2$ evolution |
| $H_2O \rightarrow Fe(CN)_6^{3-}$ | — | 25 | $O_2$ evolution |
| $H_2O \rightarrow$ FMN | + | 280 | $O_2$ uptake |
| $H_2O \rightarrow$ FMN | — | 40 | $O_2$ uptake |
| Ascorbate → FMN * | — | 187 | $O_2$ uptake |
| Catechol → FMN *,** | — | 240 | $O_2$ uptake |
| DPC → FMN *,** | — | 171 | $O_2$ uptake |
| DPC → $Fe(CN)_6^{3-}$ | — | 105 | Optical *** |
| $H_2O_2 \rightarrow$ DMQ | — | 293 | $O_2$ evolution |
| $H_2O_2 \rightarrow Fe(CN)_6^{3-}$ | — | 151 | $O_2$ evolution |

* The reaction mixture contained superoxide dismutase (Sigma; 200 units/ml) to prevent the radical oxidation of electron donors.

** The reaction mixture contained 0.5 mM D-ascorbate as electron reservoir.

*** Ferricyanide reduction was followed optically at 420 nm.

system II requires $Cl^-$ has not been answered rigorously. The oxidation of $NH_2OH$ ascorbate [5] and of $Mn^{2+}$ [25] has been shown to occur in the absence of $Cl^-$, but the significance of these results may be subject to question because the chloroplasts used in these earlier experiments were abnormal in the sense that their water-splitting machinery had been destroyed (inevitably by the $NH_2OH$ itself or deliberlately by heat-treament). The new experiments summarized in Table II clearly show that $Cl^-$-depleted chloroplasts were able, in the absence of $Cl^-$, to oxidize ascorbate, catechol, diphenylcarbazide and $H_2O_2$ at rates 4—12 times faster than that of the residual water oxidation. These reactions were highly (>90%) DCMU-sensitive, indicating an abligatory involvement of Photosystem II. Tests showed that chloroplasts pre-exposed to these reductants retained the $Cl^-$-dependent, $O_2$-evolving capability largely unimpaired. These results strongly suggest that the $Cl^-$ effect is indeed specific to the water-splitting mechanism.

*5. Effect of $Cl^-$ on sonicated chloroplasts*

The Hill reaction in sonically fragmented chloroplasts shows a $Cl^-$ concentration dependence, as shown in Fig. 5, which is kinetically very similar to that of the unsonicated chloroplasts. The magnitudes of the residual $O_2$ production, observed at $[Cl^-] = 0$, were close to what one would expect if the medium contained 0.2 mM $Cl^-$ as an impurity ($[Cl^-]_0 = 0.2$ mM in Fig. 5, inset). Although the actual level of $Cl^-$ in the "$Cl^-$-free" media used was estimated to be less

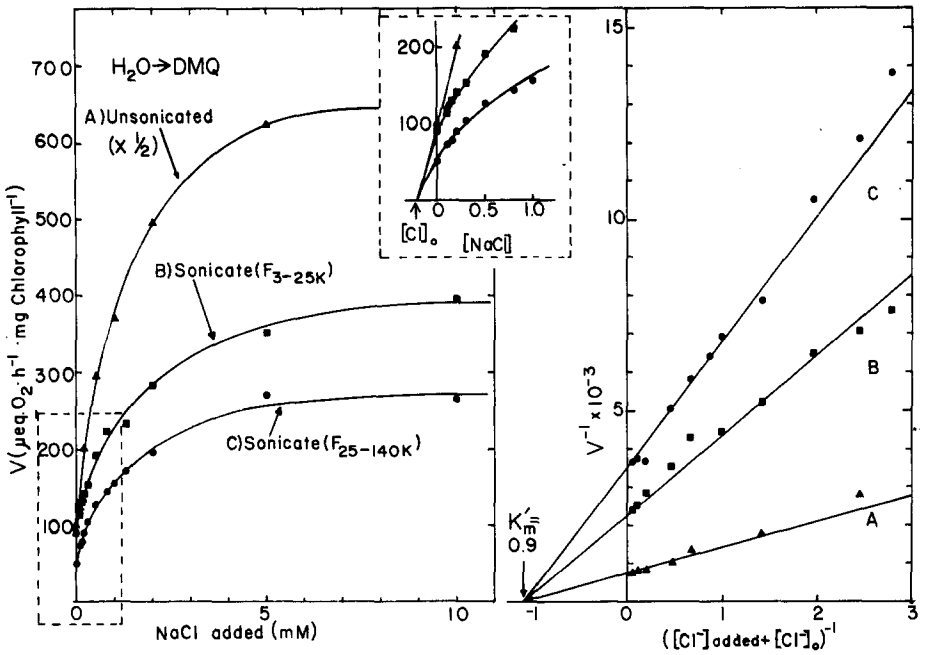


Fig. 5. The rate of $O_2$ evolution as a function of $Cl^-$ concentration in normal (unsonicated) and sonicated spinach chloroplasts with 2,5-dimethylquinone (DMQ) as the electron acceptor. $F_{3-25K}$ denotes a centrifugal fraction obtained between 3000 and 25 000 × *g*, and $F_{25-140K}$ a fraction between 25 000 and 140 000 × *g*. For details, see Methods. The reaction conditions were basically the same as for the DMQ experiment of Table I except that DBMIB was omitted in these experiments. For the explanations of the inset figure and the double-reciprocal plots, see text.

than $10^{-5}$ M (by $AgNO_3$ test), the reaction medium certainly contained some $HCO_3^-$, a weakly effective cofactor anion of $O_2$ production (see Table I). Moreover, it seems likely that the chloroplasts and subchloroplast particles used still retained significant amounts of $Cl^-$ and $HCO_3^-$ (which may be trapped in the vesicles by the fixed membrane charges). These factors may add up to an effect equivalent to that of 0.2 mM $Cl^-$. In the double reciprocal plots of Fig. 5 (right) we used $Cl^-$ concentration values which were corrected for this "basal" concentration ($[Cl^-]_0$). The plots for all three fractions display a linear dependence of $V^{-1}$ on $[Cl^-]^{-1}$ and a fixed $K'_m$ (0.9 mM). The simplest interpretation of the data is to assume that $Cl^-$ acts as a typical enzyme activator: E (inactive) + $Cl^-$ ⇌ E-$Cl^-$ (active), and that the activation constant, or apparent dissociation constant for E-$Cl^-$, is unchanged by chloroplast fragmentation ($K$ = 0.9 mM at 22°C, pH = 7.2). This enzyme activator hypothesis is further strengthened by the experiments described below.

### 6. *Effect of $Cl^-$ on the $NH_2OH$ inhibition*

In the dark $NH_2OH$ selectively attacks and irreversibly destroys the Mn-containing $O_2$-evolving center (Mn enzyme) of Photosystem II [26,27] without abolishing the ability of Photosystem II to oxidize artificial reductants [5,19]. As clearly documented in the preceding sections, $Cl^-$ removal exerts a similar "substrate-specific" inhibition of Photosystem II (except that the inhibition

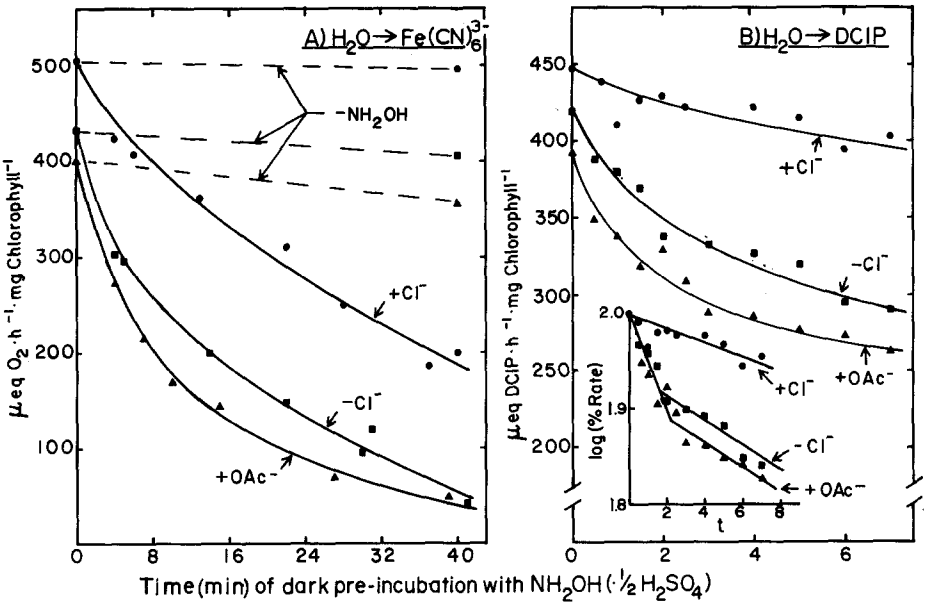


Fig. 6. The time courses of development of $NH_2OH$ inhibition in the dark in the presence and absence of $Cl^-$ (spinach chloroplasts). Ferricyanide and 2,6-dichlorophenolindophenol (DCIP) were used as electron acceptors in two separate experiments (A,B) with different batches of chloroplast preparations. Chloroplasts were incubated at 0°C in the dark with 0.4 mM $NH_2OH$ ($\frac{1}{2}$ $H_2SO_4$) in three different media: (a) medium containing $NH_2OH$ and buffer (20 mM HEPES/NaOH, pH 7.2) only, (b) medium containing $NH_2OH$, buffer and 20 mM $Cl^-$ (NaCl), and (c) medium containing $NH_2OH$, buffer, and 20 mM $OAc^-$ (sodium acetate). The concentration of chlorophyll in these preincubation mixtures was 330 $\mu$g/ml for DCIP experiments and 700 $\mu$g/ml for ferricyanide experiments. The development of $NH_2OH$ inhibition in each preincubation mixture was followed by timed samplings: Under dim room light, a small aliquot (60 $\mu$l) was taken quickly at indicated intervals and was added to a large volume (2 ml) of reaction medium which contained 40 mM HEPES/NaOH buffer (pH 7.2), 0.25 mM FeCy (or 25 $\mu$M DCIP) and excess $Cl^-$ (20 mM NaCl). To equilibrate the chloroplasts with $Cl^-$, the mixture was incubated for 2 min in the dark in the thermostated reaction chamber (22°C) and then illuminated for Hill reaction assay. $O_2$ evolution (with ferricyanide) proceeded linearly at least for 30 s and DCIP reduction 10 s. Reaction rates were computed from these initial linear phases of the reaction. The values for zero-incubation time were obtained by sampling immediately before $NH_2OH$ was added to the preincubation mixtures. The DCIP experiments were repeated twice and the data from the second set of experiments were normalized to the data from the first set at $t = 0$. The ferricyanide experiments were repeated twice using different batches of chloroplasts. The results from the second set of experiments (not shown) were very similar to those shown in Fig. 6A.

is reversible). It is therefore reasonable to suspect that the $Cl^-$-requiring step and the site of $NH_2OH$ inhibition (Mn enzyme) may be in some way closely related. In line with this notion, we now present evidence that the Mn enzyme is more resistant to $NH_2OH$ attack in the presence of $Cl^-$ than in its absence. This is not an unspecific salt effect, since acetate, which is ineffective as a cofactor of water splitting, has no protective action. In this experiment, the progress of $NH_2OH$ inhibition in the dark was followed under three different conditions: (a) $Cl^-$-free, (b) $Cl^-$-free but acetate ion present, and (c) with $Cl^-$, as detailed in the legend for Fig. 6.

The results are clear. As the $O_2$ production data for Fig. 6A indicate, the $NH_2OH$ inhibition in the dark progressed much more slowly in the presence of

$Cl^-$ ($t_{1/2}$ = 30 min) than in its absence ($t_{1/2}$ = 12 min). Acetate ion, added in lieu of $Cl^-$, failed to slow the progress of the $NH_2OH$ inhibition ($t_{1/2}$ = 8 min). Fig. 6B shows a detailed analysis of the early phases of the development of the $NH_2OH$ inhibition. In these experiments 2,6-dichlorophenolindophenol replaced ferricyanide as the electron acceptor, and the dye reduction was followed spectrophotometrically. The semilog plots of Fig. 6B (inset) reveal that the initial phase (2 min) of the development of the $NH_2OH$ inhibition was about 5 times faster when no $Cl^-$ was present in the $NH_2OH$-treatment medium than when $Cl^-$ was present therein. These results strongly indicate a direct interaction between $Cl^-$ and the target of $NH_2OH$ inhibition, namely, the Mn-containing, $O_2$-evolving center. The implication of these results will be discussed below.

## Discussion

Through the use of EDTA-washed, functionally $Cl^-$-depleted chloroplasts we have demonstrated that the DCMU-insensitive silicomolybdate reduction [13—16] does indeed require $Cl^-$. The dependence of silicomolybdate reduction on $Cl^-$ was in fact even more pronounced than in other reaction systems, in the sense that virtually no $O_2$ production occurred in the absence of added $Cl^-$ (Fig. 1). This is reminiscent of the fact that di- and trivalent anions added to the $Cl^-$-free reaction mixture further decrease the residual rate of $O_2$ production (Table I). We suspect that these anions, including the silicomolybdate anion, are not only ineffective in catalyzing $O_2$ evolution, but may also enhance the depletion of residual $Cl^-$ from the chloroplasts, for example through ion exchange or modification of the membrane state.

The silicomolybdate experiments, when taken together with the experiments involving the various artificial electron donors to Photosystem II (Table II), greatly reinforce the earlier conclusion [5,28] and leads us to the conviction that (a) the site of $Cl^-$ involvement is on the water side of Photosystem II, and (b) the step requiring $Cl^-$ is one which is specifically associated with the water-splitting mechanism. The fact that the oxidation of $H_2O_2$ by Photosystem II [29] does not require $Cl^-$ (Table II) is of special interest. Using $NH_2OH$-treated chloroplasts, Izawa and Pan [30] have recently studied the oxidation of $H_2O_2$ and obtained evidence that the protons released by the $H_2O_2$ oxidation are discharged internally, just as the protons released by the $H_2O$ oxidation are (e.g. ref. 31). Thus, the non-involvement of $Cl^-$ in the $H_2O_2$ oxidation tends to dismiss the possibility that the role of $Cl^-$ in $H_2O$ is to act as a counterion to enable the internal release of protons.

We now believe that we have obtained evidence which strongly favors the view that $Cl^-$ is a cofactor of an enzyme involved in $O_2$ evolution. Cheniae and Martin [26] have established that the bulk (70%) of the membrane-bound Mn is specifically associated with the machinery of water oxidation. Although little is known about the chemical nature of the "Mn enzyme" ($O_2$-evolving center), the enzyme is noted for its high susceptibility to denaturation by $NH_2OH$. Cheniae and Martin [26] showed that the Hill activity of chloroplasts (and of algae) exposed to 1—2 mM $NH_2OH$ in the dark at 24° C rapidly diminished following biphasic kinetics. In our dark preincubation experiments at 0° C with a

low concentration (0.4 mM) of $NH_2OH$, we found the initial fast phase of the development of $NH_2OH$ inhibition was almost completely prevented when 20 mM $Cl^-$ coexisted with the $NH_2OH$ during dark incubation. Acetate, which is inactive as a cofactor of $O_2$ evolution, had no such preventive effect (Fig. 6). The apparent antagonizing action of $Cl^-$ against $NH_2OH$ inhibition may be best explained by assuming that the cofactor action of $Cl^-$ involves the binding of the ion to the $O_2$-evolving Mn enzyme and that this ligand binding somehow alters the susceptibility of the enzyme to $NH_2OH$ attack. Similarly, the simplest explanation of the hyperbolic rate vs. [$Cl^-$] curves (Fig. 5) is to assume that a reversible $Cl^-$-enzyme binding does occur and that the binding follows the mass action law with $n = 1$. However, such a free equilibrium, implied by the kinetics, must be a result of some form of membrane modification (or "loosening") elicited by extensive washings; otherwise one would expect almost complete removal of $Cl^-$ after 1 or 2 washings, which is not the case.

Monovalent anion activation or inactivation of redox enzymes is not an unusual phenomenon (see ref. 32). In fact, the anion series reported for the inhibition of xanthine oxidase [33] and for the activation of membrane-bound succinate dehydrogenase [34] share some intriguingly similar features with the series found for the activation of $O_2$ evolution: $Cl^- > Br^- > NO_3^- \geqslant I^- \geqslant HCOO^- \geqslant HCO_3^- > F^-$ (Table I). As already pointed out, all the anion series for Hill activity found in the literature agree in placing $Cl^-$ and $Br^-$ at the top of the sequence. Discrepencies found for the sequence of the less active anions (e.g. $HCO_3^-$) may be explained, at least in part, as being due to the differences in permeability properties of the chloroplasts used by the individual workers. The relatively high permeability of the thylakoid membrane to the first 4 anions ($Cl^-$, $Br^-$, $NO_3^-$, $I^-$) of the above series is well documented [35,36]. However, the anion permeability properties of EDTA-uncoupled chloroplasts have yet to be investigated. It is of interest to add here that our experiments with EDTA-washed, $Cl^-$-depleted chloroplasts revealed a weak cofactor action of $HCO_3^-$ on $O_2$ evolution. This $HCO_3^-$ effect is presumably unrelated to Warburg's $CO_2$ effect ($CO_2$ or $HCO_3^-$ requirement of the Hill reaction [37]), an effect which is now believed to occur near the primary electron acceptor of Photosystem II [38]. Nevertheless, the existence of two sites of $HCO_3^-$ interaction near Photosystem II, one before and one after the photosystem, is highly intriguing.

During the preparation of this report, Terry [39] has published a paper which deals in part with the relation of the $Cl^-$ content to the Hill activity of sugar beet chloroplasts. His data indicate that sucrose washing and EDTA washing were equally effective in terms of $Cl^-$ removal but the $Cl^-$ effect was much less pronounced in the sucrose-washed chloroplasts (2—3-fold stimulation in the sucrose-washed chloroplasts as opposed to 5—10-fold in the EDTA-washed chloroplasts). Thus, Terry questions the physiological significance of the $Cl^-$ effect observed in EDTA-washed chloroplasts. However, his data (Table III of ref. 39) show that the $Cl^-$-stimulated rate of ferricyanide reduction in the sucrose-washed chloroplasts was much lower than in EDTA-washed chloroplasts. This suggests that his sucrose-washed chloroplasts were still coupled, and consequently, the ferricyanide reduction rates (assayed without uncouplers) were limited by the energy coupling mechanism. Furthermore, $Cl^-$

analysis for stock suspensions of chloroplasts * may not necessarily reflect the $Cl^-$ content of the chloroplasts after they have been transfered to the higher temperature reaction media. Since the membrane diffusion processes would now be greatly accelerated, the degree of $Cl^-$ depletion actually attained in the reaction mixture might be quite different depending on the state of the membrane.

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* The $Cl^-$ content data Terry gives for chloroplasts in Table III (ref. 39) seem to be in error. The data indicate that >90% $Cl^-$-free chloroplasts still contain up to 30 μmol $Cl^-$ per mg chlorophyll. This value corresponds to a $Cl^-$ concentration in the chloroplasts as high as 0.3 to 3 M, if one makes a reasonable assumption that the chlorophyll concentration in the chloroplasts is between 10 mM and 100 mM (cf. ref. 40). It is interesting to note that the recent neutron activation experiments of Nakatani and Barber [41] indicate that even in intact chloroplasts (pea, spinach) the $Cl^-$ content is only 0.1 to 0.2 μmol/mg chlorophyll.

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