



Minireview

## Chloride and calcium in Photosystem II: from effects to enigma\*

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Received 4 July 2001; accepted in revised form 14 November 2001

**Key words:** Daniel Arnon, Gerald Babcock, calcium, chloride, Seikichi Izawa, Photosystem II, Otto Warburg, water oxidation

### Abstract

This minireview focuses on the early evidence for roles of chloride and calcium ions in reactions of photosynthetic electron transport and on the reluctance with which an essential function of these inorganic ions in the process of water oxidation was accepted. For example, Daniel Arnon's group initially refuted the conclusion of Otto Warburg, the discoverer of a 'chloride effect,' that chloride was a 'coenzyme.' Their reasoning was that chloride had not been shown to be an essential mineral nutrient of plants. In the case of calcium, the problem was that the first 'calcium effects' had been seen with preparations from cyanobacteria rather than from green plants. While today the status of calcium and chloride as essential participants in the process of water oxidation is rarely disputed, the nature of their involvement still eludes all experimental inquiries. Substantial progress in this respect may come from recent refinements of the application of Fourier-transform infrared spectroscopy to the study of photosynthetic water oxidation.

### Introduction

Refined and new instrumentation, innovative biochemical methodology, computerized data management, and the emergence of DNA technology have revolutionized biological research during the last two decades. In the wake of these developments, the questions we ask have become ever more incisive and our experimental inquiries more sophisticated while past research often takes on an aura of triviality and irrelevance. This historical minireview of research on the roles of chloride and calcium in the mechanism of photosynthetic water oxidation provides me with an opportunity to describe how the contributions of earlier investigators, including those of the pioneers, have shaped the course of discoveries and the evolution of our thinking. I will reflect on the conceptual chal-

lenges these scientists, some of them almost forgotten, struggled with, and how perceptive their conclusions often were. With such a focus it will be impossible to do justice to the myriad of more recent contributions. My selection of relevant publications, therefore, must be haphazard and subjective, but I can refer the reader to the excellent comprehensive reviews in a book edited by Donald Ort and Charles Yocum (1996) and to up-to-date accounts in a special issue of *Biochimica et Biophysica Acta*, edited by Jonathan Nugent (2001).

### Chloride – protectant or cofactor?

The history of research on the function of chloride in photosynthetic oxygen evolution began in 1944 when Otto Warburg and Wilhelm Lüttgens published their discovery of a beneficial effect of chloride on the Hill reaction of fragmented chloroplasts. Only a few other monovalent anions were found to serve as substitutes for chloride with various degrees of effectiveness. Two years later, the authors assigned to chloride the status

\* I dedicate this minireview to the memory of George Cheniae (1928–2001), my colleague for almost 40 years, whose excellence as a scientist and fairness have been an inspiration, whose critical mind I admired, whose wit I enjoyed, and whose friendship I cherished.

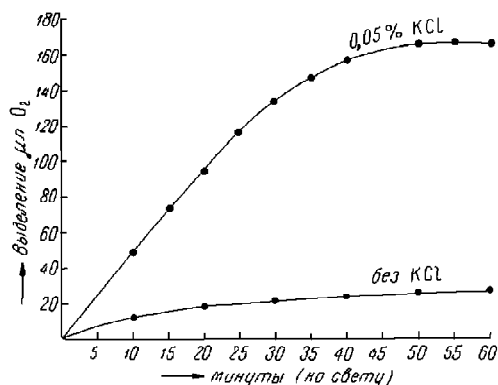


Figure 1. First published evidence of a stimulatory effect on the Hill Reaction in the 1946 *Biokhimiya* article of Otto Warburg and Wilhelm Lüttgens. Translation of legend and inscription of axes: reactivation of washed granules by an addition of chloride. One  $\mu$ mole chlorophyll per 2 ml granule suspension in 1/20 M phosphate buffer pH 6.5. 20°. Gas phase argon. At  $t_0$  addition of 2 mg quinone in 0.2 ml N/100 H<sub>2</sub>SO<sub>4</sub> and beginning of illumination. Ordinate: O<sub>2</sub> evolution in  $\mu$ l; abscissa: minutes (in light). Upper trace: 0.05% KCl present; lower curve: without KCl.



Figure 2. Otto Warburg in his laboratory [photograph scanned from the biography of Otto Warburg by Krebs (1979); reproduced with permission].

of a coenzyme of the photosynthetic light reactions. This was done in an article (Warburg and Lüttgens 1946) that is a historical document because of its scientific content, and a historical curiosity because it was written in Russian for the journal *Biokhimiya* of what was then the Soviet Union. From the section 'Koferment' I reproduce in this review the graph in which a stimulatory effect of chloride on the Hill reaction is documented for the first time (Figure 1; Figure 2 shows a photograph of Warburg in his laboratory). Doing so, I also must comment on the circumstances that gave us a paper by Otto Warburg in Russian. In 1930, Warburg had received funds from the Rockefeller Foundation to establish the Kaiser-Wilhelm-Institut für Zellphysiologie for his research activities. It was built in Dahlem, a district of Berlin which at the end of the second World War became part of the city's American sector. Warburg, however, found himself under the rule of Soviet occupation forces. In 1943, when air raids made Berlin a dangerous place to live, he had accepted an offer by Prince Eulenburg to relocate staff and equipment to his *Schloss* (castle) *Liebenberg* in the Brandenburg countryside north of the city. In a biography of Otto Warburg, Hans Krebs (1979) tells us that all of Warburg's laboratory equipment and supplies were confiscated by the Soviets and hauled away (see also Dean Burk 1953). Soon after, Warburg was assured by Marshal Zhukov, the Soviet Commander-in-Chief, that this had been an unfortunate error that would be corrected, but it never was.

Perhaps Warburg's decision to publish his article in Russian was part of his strategy to regain possession of the institute's property. Indeed, the authors' address makes it appear as if a Kaiser-Wilhelm Institut für Zellphysiologie, Liebenberg still existed (reference to 'castle' must have been deemed inappropriate by the Soviet editors).

Many years would pass before the question of a critical role of chloride in photosynthetic electron transport became a real issue even though other research groups provided much additional insight soon after the publication of Warburg and Lüttgens' studies. When my own history in the field of photosynthesis began in the laboratory of the eminent Hans Gaffron (Figure 3), his comprehensive review of photosynthesis (Gaffron 1960) became for me an indispensable source of wisdom. I decided, therefore, to revisit his article and find out what he had to say about chloride 15 years after Warburg and Lüttgens' report. Not much, I had to conclude after finding chloride mentioned only in a quote inserted into the discussion of the Hill reaction: 'Damaged and fragmented chloroplasts are stabilized by potassium chloride but not by media of high osmotic pressure. Whole chloroplasts are stabilized by media of high osmotic pressure (sucrose solution), but not by chloride.' But where exactly was this quote taken from? It reflects the spirit of Thomas Punnett's conclusions in a paper of 1959, but may have been taken from one of Punnett's 1956



Figure 3. The master and his apprentice: Hans Gaffron and the author of this minireview in the mid-1960s (photograph by Govindjee).

articles which I did not have access to (however, see Chem Abstr. 51, 5906d).

The perception of chloride as a protectant of photosynthetically active *in vitro* systems from damaging effects of temperature and light and from deterioration during storage had emerged from the post-Warburg investigations by Daniel Arnon and Bob Whatley (1949a, b) and by Paul Gorham and Kenneth Clendenning (1952). To Arnon and Whatley (1949a), calling chloride a coenzyme, as Warburg and Lüttgens (1946) had done, was heresy. They argued that the underlying proposition was ‘rather striking’ because it would represent ‘the first instance in the history of plant nutrition where the essentiality of an inorganic nutrient was established by the discovery of its biochemical function, in the absence of corroborative evidence from growth experiments according to specific criteria of indispensability.’ Actually, a beneficial effect of chloride on plant growth had been shown by Charles Lipman as early as 1938, but Arnon and Whatley found that, ‘as expected,’ sugar beet and chard plants ‘made excellent growth in media to which no chloride was added.’

In the 1960s, the reputation of chloride improved. Ironically, it was Arnon’s group (M. Losada et al. 1961) that added chloride to the water-oxidizing step in Hill and Bendall’s Z-scheme soon after its debut. What had turned chloride’s fortune? As Arnon and his coworkers explained in a 1963 article (J.M. Bové et al. 1963), their change of mind began when they learned that other laboratories had shown chloride to be an essential micronutrient of green plants. From among the nutrition studies, the one by G. Martin and J. Lavollay (1958) with *Lemna* deserves mention because a very painstaking purification of the nutri-

ents allowed these authors to establish a beneficial effect of as little as 0.5  $\mu\text{M}$  chloride in the growth medium. Arnon’s research team then undertook a systematic comparative analysis of the optimal conditions for various photosynthetic reactions including cyclic and noncyclic photophosphorylation and established that only reactions depending on a functional water oxidizing system benefited from a presence of chloride in the reaction medium (Bové et al. 1963). The team conceded that chloride ‘is a coenzyme of oxygen production in photosynthesis’ but the collected experimental data did not yet justify assigning chloride to the water oxidation step of the Z-scheme as had been done in the earlier paper (Losada et al. 1961). Proof for such a localization was eventually provided by Geoffrey Hind, Seikichi Izawa, and their collaborators (Hind et al. 1969; Izawa et al. 1969) in a thorough and critical inquiry into the nature of the impairment of electron transport in isolated chloroplast thylakoids that displayed a ‘chloride effect.’

These revelations should have added a new perspective to research on the mechanism of photosynthetic water oxidation, but this was not to happen for at least another decade. An aura of artificiality still tainted the conditions that were necessary to observe a response to added chloride. The requirement of neutral or slightly alkaline media (Gorham and Clendenning 1952) had been rationalized by Hind et al. (1969) with a need to neutralize positively charged groups of proteins which tie down the native chloride. But they added another conundrum: they could elicit large chloride effects only with uncoupled thylakoid preparations. Hind et al. rejected the possibility that the low ‘basal’ rates remaining under coupled conditions were supported by residual small amounts of chloride because lower, chloride sensitive rates could be obtained after addition of an uncoupler. Instead, they invoked synergistic inhibitory consequences of chloride deficiency and actions of the uncoupling agents. However, ten years later, the dissertation research of Steve Theg in my laboratory would tell a different story (Theg and Homann 1982). Of course, Steve’s research was done with some much-improved knowledge about the water-oxidizing complex. Most importantly, it had been established in the meantime that the site of water oxidation was on the lumen side of the thylakoid membrane. It turned out that the low permeability of the thylakoid membrane to protons allows the pH at the site of water oxidation to remain sufficiently acid to prevent the dissociation of functional chloride even in slightly alkaline suspension media. This explained

why all previous investigators had seen a significant chloride requirement only when, for one reason or another, the thylakoid membranes were in a condition that rendered them leaky to protons.

Today, the preoccupation of early investigators with the significance of the treatments necessary to achieve a 'chloride effect' is perhaps nothing more than a historical curiosity. We are now accustomed to using Photosystem II (PS II) preparations, typically of the type introduced by Deborah Berthold, Gerald Babcock and Charles Yocum (1981), which provide unimpeded access to the water-oxidizing complex. Indeed, our present state of knowledge about the role of chloride could not have been achieved without the availability of such experimental material. Its usefulness, however, makes us forget perhaps too easily that we work with a water oxidizing complex that is exposed to an artificial medium instead to its *in vivo* environment, the thylakoid lumen.

As I explained at the beginning of this review, for my account of more recent investigations of the chloride problem I will refer to a selection of just a few contributions. I begin with the elegant studies by Seikichi Izawa and his colleagues (Muallem and Izawa 1980; Muallem and Lainé-Böszörményi 1981), still performed with thylakoid preparations, in which the first evidence was provided for an abnormal oxidant storage in the water-oxidizing complex after chloride removal. The unusually long-lived oxidants discovered by these investigators were subsequently detected by others as well and assigned to the  $S_2$ -state of 'Kok's clock' (Kok et al. 1970), which describes the oxidant accumulation in the oxygen-evolving complex. As it would turn out, in chloride-depleted water-oxidizing complexes the  $S_2$ -state is a dead end in the progression of oxidant storage. Proof of the participation of chloride also in the dioxygen producing transition from  $S_3$  to  $S_0$  was provided only recently by Hanna Wincencjusz, Hans van Gorkom and Charles Yocum (1997), but had been postulated already as early as 1984 by John Sinclair.

The revelations regarding the  $Cl^-$  requirement in the progression of light-induced oxidant accumulation have told us where to look for chloride actions, but an understanding of the nature of these actions still eludes us. It is not surprising, therefore, that chloride still seems to enter most models of the mechanism of photosynthetic water oxidation as an afterthought. In their insightful analysis of chloride action on electron transport, Gorham and Clendenning (1952) had envisaged a charge-regulating role of chloride. Refinements

of this concept by William Coleman and Govindjee (1987) and by us (Homann et al. 1983) invoked a critical role of the charge distribution on proteins in the proper conduction and disposal of the protons that are liberated during the S-cycle. An alternative proposed function for chloride as an electron-transferring bridging ligand in the manganese cluster goes back to a suggestion made already in 1963 by Bové et al. and has been elaborated upon in a review by Christa Critchley (1985). Prompted by the observation that the easily oxidizable iodide ion can assume the function of chloride rather effectively, we questioned several years ago whether a close proximity of the activating anion to the oxidized Mn-cluster actually is a realistic proposition (Rashid and Homann 1993). However, in the meantime, Charles Dismukes has explained to me that as an inner sphere ligand in the company of other ligands such as oxide, activating iodide might be resistant to oxidation by manganese in elevated oxidation states. Perhaps so, yet several recent models assume the activating anion to hop into and out of the coordination sphere of manganese during the S-cycle.

Many years ago, my dear colleague Sei Izawa remarked to me that 'plants do not split water, they split sea water.' His intriguing perception was inspired by actual determinations of the amounts of chloride found in washed, chloride sufficient thylakoids (Kelley and Izawa 1979) but seems to be without basis now that we have been persuaded by more recent analyses (Lindberg et al. 1993) that each water-oxidizing complex may require only a single chloride. Yet, it remains a puzzling reality that more than 0.1 M chloride must be provided in the suspension medium to maintain the integrity of the Mn cluster in membrane preparations from which the Mn-stabilizing 33-kDa polypeptide has been removed (Miyao and Murata 1984b). Perhaps the Cl requirement of the water-oxidizing complex is indeed a reminder that the primordial Mn cluster was designed with the help of high concentrations of chloride. The acquisition of the 33-kDa polypeptide then obviated critical structural functions of chloride and allowed an escape from the original high-chloride environment.

### Calcium, manganese's unexpected partner

Calcium has been a latecomer in the models of photosynthetic water oxidation. The redox functions of the water-oxidizing center seemed to be served well by manganese, and there was no use for another metal

ion, certainly not for one that was redox-inactive. Thus, little attention was paid to Walter Fredricks and André Jagendorf's discovery in 1964 of a 'calcium effect' on photosynthetic electron transport in a cyanobacterial preparation. This did not change much when, a decade later, Richard Piccioni and David Mauzerall (1976) followed up on that observation and concluded that the stimulatory action of  $\text{Ca}^{2+}$  was 'close to the point of water oxidation.' Presumably, the observed effects were dismissed as peculiarities of cyanobacteria, especially because they were not entirely specific for  $\text{Ca}^{2+}$  but could be mimicked to some extent by  $\text{Mg}^{2+}$  (Brand 1979). Yet, at the time of the studies of Piccioni and Mauzerall,  $\text{Ca}^{2+}$  had been reported already by Takashi Yamashita and Giiti Tomita (1975) to be one of the essential ingredients for a reactivation of water oxidation in thylakoid preparations from green plants after removal of the functional manganese. The first evidence for a stimulatory effect of calcium on electron transport in thylakoids from a higher plant apparently was not published for another seven years. It came finally from Govindjee's laboratory in a study of the photochemical properties of preparations from mangrove leaves (Critchley et al. 1982). Nonetheless, a role of  $\text{Ca}^{2+}$  in water oxidation became an issue only after investigations began focusing on inhibitory treatments of chloroplast-derived thylakoids (Barr et al. 1983) and of PS II membranes (Ghanotakis et al. 1984; Miyao and Murata 1984a) that could be reversed quite specifically by  $\text{Ca}^{2+}$  ions. Only  $\text{Sr}^{2+}$  could substitute for  $\text{Ca}^{2+}$  in these experiments, but with a much lower effectiveness.

The road to an acceptance of calcium as a cofactor of the water oxidation was shorter than that for chloride and not quite as bumpy. Even so, it also began with doubts about an essential role. For example, Mitsue Miyao and Norio Murata (1984a) had argued initially that added calcium might satisfy requirements usually provided by the extrinsic 24-kDa polypeptide because in experiments with PS II membranes a stimulation of the water oxidation by calcium was contingent upon a prior removal of this polypeptide. Another contentious issue was how to reconcile measured water-oxidizing activities and calcium abundances in preparations from chloroplasts differing in their content of the light-harvesting chlorophyll protein complex II (LHCP II). At the root of the ensuing controversy was an apparent heterogeneity of the calcium complement in PS II preparations from green plants. Even though some of the disputed experimental facts still may have to be clarified (cf. Boussac and Rutherford

1992; Han and Katoh 1993), general agreement has emerged that native PS II units contain a finite small number of bound calcium ions (Kashino et al. 1986; Cammarata and Cheniae 1987; Ono and Inoue 1988) of which one functions in the mechanism of photosynthetic water oxidation. Another PS II-associated  $\text{Ca}^{2+}$  ions was localized by Kab-Cho Han and Sakae Katoh (1993) on LHCP II, which Dan Davis and Elizabeth Gross (1975) had shown two decades earlier to provide high-affinity binding sites.

As in the case of chloride, the actual contribution of calcium to the mechanism of water oxidation remains a matter of speculation. Removal of either cofactor results in abnormalities of oxidant storage and progression which, however, are unique for each of them. A bond between  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  is sometimes invoked, but evidence for it is equivocal. A plausible function of calcium may be an involvement in the access and/or delivery of substrate water to the Mn cluster as initially proposed by William Coleman and Govindjee (1987), Charles Dismukes and collaborators (Sivaraja et al. 1989), and William Rutherford (1989). The proper positioning of calcium at the active site may be accomplished by being attached to a carboxylate group on a protein moiety (Noguchi et al. 1995). Such a location of calcium would be consistent with an early observation by Christine Yerkes and Gerald Babcock (1984) of calcium-specific effects on redox reactions in a manganese-depleted PS II. Interestingly, structural ramifications of the presumed binding of calcium to a protein extend beyond the water-oxidizing complex to the opposite side of the membrane where the redox potential of the primary acceptor  $\text{Q}_A$  is affected (Krieger et al. 1993). Regarding our perception of the function of calcium, an exciting new dimension has been added by Anja Krieger and Engelbert Weis (1993), who provided evidence for a regulation of PS II activity *in situ* by a reversible dissociation of functional  $\text{Ca}^{2+}$  when the thylakoid lumen becomes over-acidified under photo-inhibitory conditions. This suggested physiological significance of PS II-associated calcium undoubtedly will receive further attention.

### Beyond the inorganic Mn–Cl–Ca core

For quite a while now we have abandoned the notion that photosynthetic water oxidation can be understood as a reaction performed by a cluster of four oxygen-bridged manganese atoms in various oxidation states.

First came the revelation of a probable participation of chloride, then calcium entered the picture, and now we take into account the cooperation of the manganese center with amino acid residues nearby, e.g., the redox-active tyrosine  $Y_Z$  and histidine-190 on the D1 protein. Reports of structural ramification of chloride binding and the implication of a carboxylate group as a linkage between functional calcium and the manganese center have drawn the circle wider yet. Thus, structural features of the proteins surrounding the inorganic core and their spatial relations are recognized to be critical for the orderly succession of events leading to the oxidation of two water molecules. This includes the light-driven oxidant accumulation, the delivery and binding of substrate water, the formation and disposal of dioxygen as well as the management of the released protons.

An exciting first glimpse at the structural dynamics accompanying the progression of oxidant accumulation has been provided very recently through two independently conducted analyses of flash induced changes of Fourier-transform infrared spectra, one performed by Tokumi Noguchi and Miwa Sugiura (2001), the other by Warwick Hillier and Gerald Babcock (2001). This extension of our view beyond the redox-active constituents of the water-oxidizing complex to the surrounding proteins has set the stage for a new and revealing look at the still enigmatic functions of chloride and calcium. It is fate's tragic irony that Jerry Babcock helped to usher in this new era of PS II research, but has been denied to take part in it.

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