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Minireview

Period-four oscillations of the flash-induced oxygen formation in photosynthesis*

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Abstract

In this minireview, the earlier researches that led to the discovery of the period-four oscillations of the flash-induced oxygen formation are presented. It also includes the background of the classical model proposed by Bessel Kok, in which the formation of oxygen requires the sequential accumulation of four positive charges on the donor side of the same reaction center.

Beginning with the discovery of photosynthesis by Joseph Priestley in the 1770s (Priestley 1772), and up to the middle of the 20th century, most of the information available on the photosynthetic process was obtained from the measurements of gas exchange, oxygen or carbon dioxide, between algae or plants and the external medium. From such a limited source of information, only formal interpretations involving unidentified chemical compounds were developed. Nevertheless, during this 'black-box' era, a number of important questions on the mechanism of photosynthesis were addressed, which led to mechanistic and structural interpretations that remain at the basis of modern photosynthesis (Rabinowitch 1945, 1951, 1956).

A landmark in our discipline is the classical experiment of Robert Emerson and William Arnold (1932a, b), who measured the amount of oxygen evolved by short saturating flashes given to a suspension of the unicellular alga *Chlorella* (see Myers 1994). The slow time response of the Warburg apparatus used in these experiments made only possible the measurement of the average oxygen yield per flash, but despite this technical limitation, this work opened a new era in the studies of the photosynthetic apparatus. The major conclusion was that upon illumination by flashes shorter than 10^{-4} s, the maximum amount of oxygen evolved per saturating flash is ~2500 times lower than the concentration of chlorophyll (Chl). The conclusions of Emerson and Arnold were difficult to reconcile with the high quantum yield of the photosynthetic process, which implies that most of the photons absorbed by Chl molecules are able to induce photochemistry. In the following decade, two classes of interpretations were proposed to interpret the data of Emerson and Arnold. These interpretations differ in terms of the structural organization of the photosynthetic apparatus.

James Franck and K.H. Herzfeld (1941) proposed a 'biochemical' hypothesis involving the diffusion of a high-energy intermediate. They assumed that the absorption of a photon by any Chl molecule leads to the formation of a freely diffusing unstable photoproduct P. This implies that a short saturating flash induces the formation of a number of P equal to that of Chls. Franck and Herzfeld proposed that P be stabilized at the level of the Emerson and Arnold enzyme E, present

* This paper is dedicated to René Wurmser who guided me towards the study of photosynthesis. René Wurmser (1890–1993), a pioneer researcher in photosynthesis, proposed in 1930 that the primary photochemical reaction must be coupled with the photolysis of water (Joliot 1996).

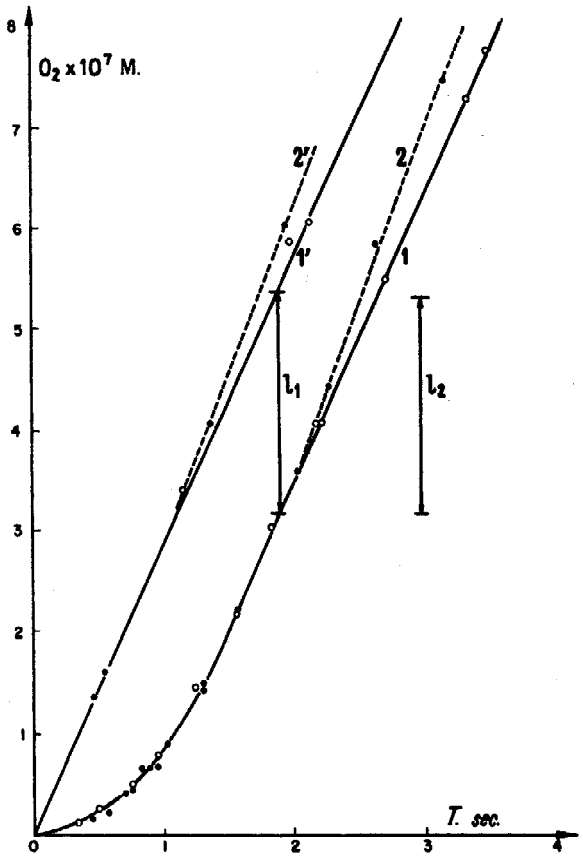

Figure 1. Kinetics of oxygen emission at the onset of a weak illumination detected with a 'stirred' electrode. *Chlorella* suspension is submitted to pulses of weak light. The amount of oxygen evolved is plotted as function of the duration of the pulse. This method eliminates the time delay associated with the time response of the apparatus or with the dark reactions that led to oxygen formation. Curve 1: dark-adapted algae. λ = 650 nm. Curve 1′: dark-adapted algae preilluminated by a short flash given 1 s before the pulse of weak light. Curves 2 and 2′: same as curves 1 and 1′ but for λ = 695 nm. l_1: deficit of oxygen formation associated with the activation reaction. l_2: Amount of oxygen produced by a single flash given to algae preilluminated by 1 flash. 695 nm and 650 nm light intensities have been adjusted in order to obtain the same rate of oxygen emission in preilluminated algae. Reproduced from Joliot (1965).

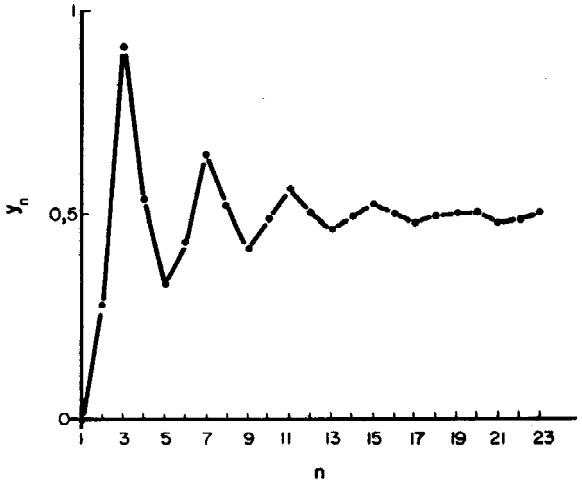

Figure 2. Oxygen evolved (y_n) by a series of saturating flashes (n) given 300 ms apart to dark-adapted suspension of green alga *Chlorella.* Reproduced from Joliot et al. (1969).

at a much lower concentration than Chls. If one assumes that the turnover of E is slower than the lifetime of P, the amount of P stabilized per flash is equal to the concentration of the Emerson and Arnold enzyme E. On the contrary, under weak-light excitation, all of P is stabilized, leading to high quantum yield efficiency.

In 1936, Hans Gaffron and K. Wohl had already proposed a hypothesis based on the physical concept of excitation energy transfer, developed by Francis Perrin (1932) and later by Theodor Förster (1948). (A photograph of Franck and Gaffron can be viewed in Homann, this issue.) Gaffron and Wohl suggested that pigment molecules were so closely packed that the excited state localized on a pigment could be rapidly transferred to neighboring pigments. According to this process of resonance transfer, excitation energy moves randomly within the pigments until it reaches a trap where the photochemical energy conversion occurs. In this model, the traps for excitation energy, equivalent to the reaction centers, are present at a concentration of the Emerson and Arnold enzyme E. The process of transfer is completed in a time shorter than the lifetime of Chl fluorescence. In this new concept, long-distance energy transfer has no obligatory requirement for movement of molecules but can occur in a solid state-like structure, provided that the distance between pigments is small enough. Thus, Gaffron and Wohl had already introduced the concept of 'solid state' biology that has been later applied to the mechanism of electron transfer within cofactors included in protein complexes. Louis N. M. Duysens (1952), who demonstrated, in his doctoral thesis, that efficient energy transfer occurs between pigments in photosynthetic bacteria, plants and algae, and experimentally established the validity of the theoretical concept of Gaffron. (See Govindjee et al., this issue, for a photograph of Duysens, as well as the cover of his doctoral thesis.)

In 1956, Eugene Rabinowitch pointed out that the concentration of the Emerson and Arnold enzyme E is not 1/2500 of the Chl concentration, but has to be multiplied by n or n/2, n being the quantum requirement

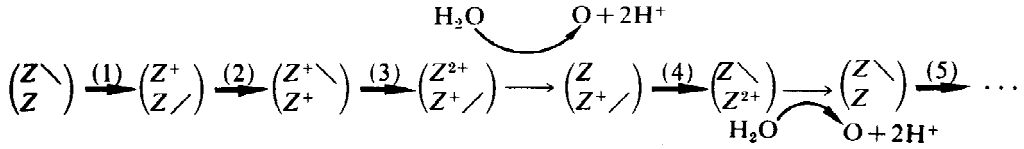

Figure 3. Charge accumulation model for the formation of oxygen. 'Z' stands for an electron donor of Photosystem II. Reproduced from Joliot et al. (1969).

of photosynthesis. Rabinowitch proposed 'the catalyst might have to operate n or n/2 times in the reduction of one CO_2 molecule and the liberation of one oxygen molecule.' This led Rabinowitch to a correct estimation (1 center for 300 Chls) of the concentration of the reaction centers that trap the excitation energy.

It was assumed that a single photoreaction could extract only one electron or one proton from water. A key problem, not addressed by Gaffron and Wohl, was to understand how four photoreactions cooperate in order to split two water molecules and to form one oxygen molecule, according to the equation:

$$2H_2O \rightarrow O_2 + 4H.$$

Progress in this field was made possible by the improvement of the techniques of oxygen detection, both in terms of time response and sensitivity. James Franck et al. (1945) developed a technique based on the measurement of the quenching of phosphorescence by oxygen that allows the detection of oxygen emission induced by a single flash. This method requires that no oxygen be present in the suspension medium, and Franck et al. (1945) observed that, in the case of dark-adapted algae, several flashes were required to induce oxygen formation. In these experiments, the O_2 evolved never exceeded 1/10000 of the Chl concentration, even when using flashes as long as 30 ms. This value was 5 times lower than the yield measured by Emerson and Arnold with much shorter flashes but in aerobic conditions. We know now that in anaerobic conditions most of the primary and secondary acceptors of Photosystem (PS) II are in their reduced state, thus explaining the low yield measured by Franck and coworkers.

The author (Joliot 1956) developed a new highly-sensitive amperometric technique for oxygen detection. A suspension of algae is placed in a cylindrical thin cuvette. A flat disc, rotating at 3000 rpm, homogenizes the oxygen concentration. Experiments were performed in the presence of oxygen in order to avoid the inhibitory effect induced by anaerobiosis. The time resolution of the method (∼0.1 s) permitted a clear discrimination between oxygen formation, oxygen uptake and respiration. A lag phase, of much shorter duration than that reported by Franck et al. (1945), was observed when dark-adapted algae were submitted to a weak continuous illumination. The duration of this lag phase is inversely proportional to the light intensity, thus showing that a priming photochemical process was involved (Joliot 1961a, b). Illumination by a single short saturating flash does not lead to any oxygen formation but abolishes the lag phase observed at the onset of a continuous illumination. P. Joliot observed that the yield measured on the second flash was close to that measured in steady state conditions and was proportional to the concentration of the Emerson and Arnold enzyme. The high oxygen yield observed on the second flash is now explained by the long duration (half time ∼50 μs) of the flashes that were used in these early experiments. Even at 0 °C, the probability of 'double hitting' was close to 30%, thus inducing a large oxygen release on the second flash. It was concluded that after dark adaptation, the Emerson and Arnold enzyme E was in an inactive state. The absorption of a first photon was required to place the enzyme in its active state, able to evolve oxygen.

C.P. Whitthingham and P.M. Bishop (1961) reported that a long dark period (1 s) between the two first flashes was required to observe oxygen evolution, a time much longer than what P. Joliot had measured. (A photograph of Whittingham appears in Walker, this issue.) The authors interpreted these data in the frame of the new concept of two photoreactions working in series, which was emerging at this time (see Govindjee, 2000, for a historical account). Further, the authors concluded that oxygen evolution requires the sequential involvement of PS I and PS II reactions. This conclusion is correct if we remember that the experiments of Whitthingham and Bishop, as those of Franck and coworkers, were performed in anaerobic conditions, which induce the reduction of the plastoquinone pool. In these conditions, several PS I turnovers are required to oxidize the pool of primary and secondary PS II donors and to activate PS II.

Joliot (1965) compared the action spectra of the priming process to that leading to the oxygen release.

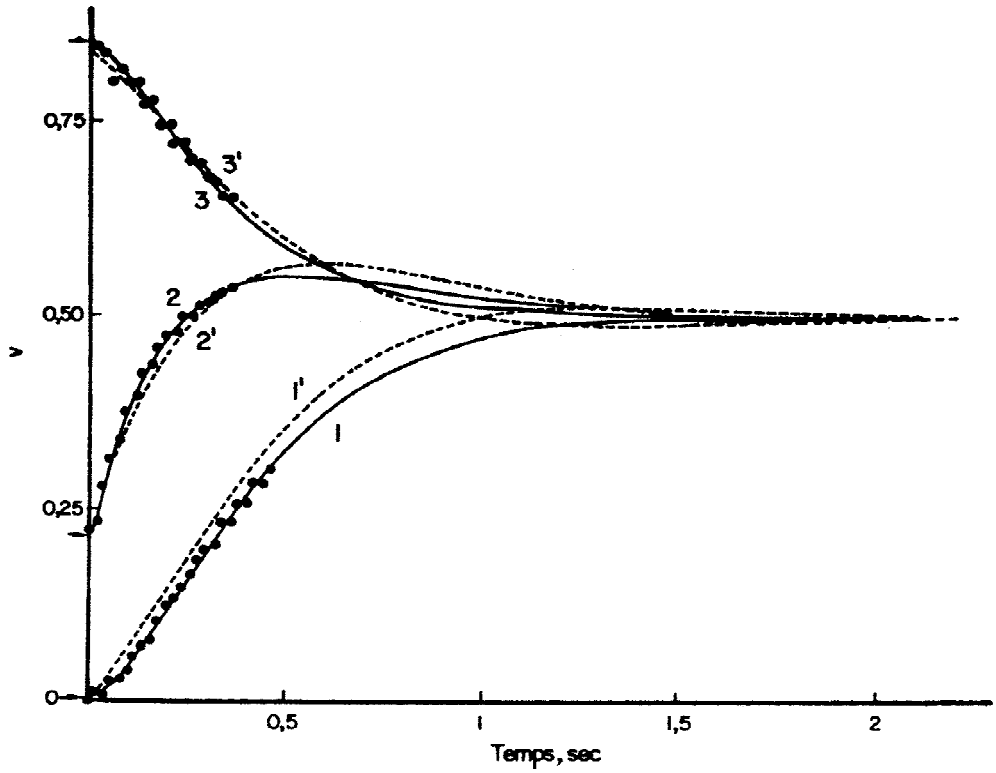

Figure 4. Kinetics of oxygen evolution at the onset of the illumination: modulated electrode. Rate of oxygen evolution plotted as a function of time ('temps' in French). (Commas are used instead of periods in French: when writing 0.1 s, one uses 0,1 s.) Curve 1: dark-adapted algae. Curve 2: algae preilluminated by 1 flash. Curve 3: algae preilluminated by two flashes. Dotted lines (numbered as 1′, 2′ and 3′): simulation based on the model shown in scheme 1. Reproduced from Joliot et al. (1969).

Figure 1 clearly shows that the priming process at the origin of the lag and the reaction that leads to oxygen evolution are both sensitized by pigments associated with PS II. This experiment demonstrates that the activation reaction studied by Joliot is a different process than that studied by Franck and coworkers and later characterized by Whittingham and Bishop.

Joliot (1968), using flashes of shorter duration, showed that a maximum amount of oxygen is evolved not on the second but on the third flash of a series; Joliot developed a new amperometric method allowing the measure of the average rate of oxygen release under a weak modulated light. This method, in which algae are settled on a bare-platinum electrode, was later applied with success to the detection of oxygen evolved under flash excitation. The sensitivity of this method is much higher than that of the method where cell suspensions are stirred; this is due to the high concentration of algae or chloroplasts present in the thin layer in direct contact with the platinum electrode. Using this 'modulated electrode,' Joliot (1968) demonstrated that the quantum yield measured at the onset of a continuous illumination following a preillumination by two flashes is about two times higher than the steady-state quantum yield of photosynthesis. P. Joliot was thus led to propose the first charge-accumulation model. In this model, the formation of one oxygen atom requires two sequential photoreactions. Joliot, G. Barbieri and R. Chabaud (1969) further observed that upon illumination of a *Chlorella* suspension by a series of short flashes, oxygen evolution oscillates with a characteristic period of four (Figure 2). This experiment was performed using the stationary bare-platinum electrode, which has a much higher sensitivity than the method in which the sample is stirred over the electrode. This experiment was interpreted according to scheme 1 (Figure 3) . In this model, the formation of one oxygen atom requires a sequential accumulation of two charges on an electron donor Z (first step in the two-step 'memory'). In addition, two secondary donors Z are associated with the reaction center, alternately connected to the primary

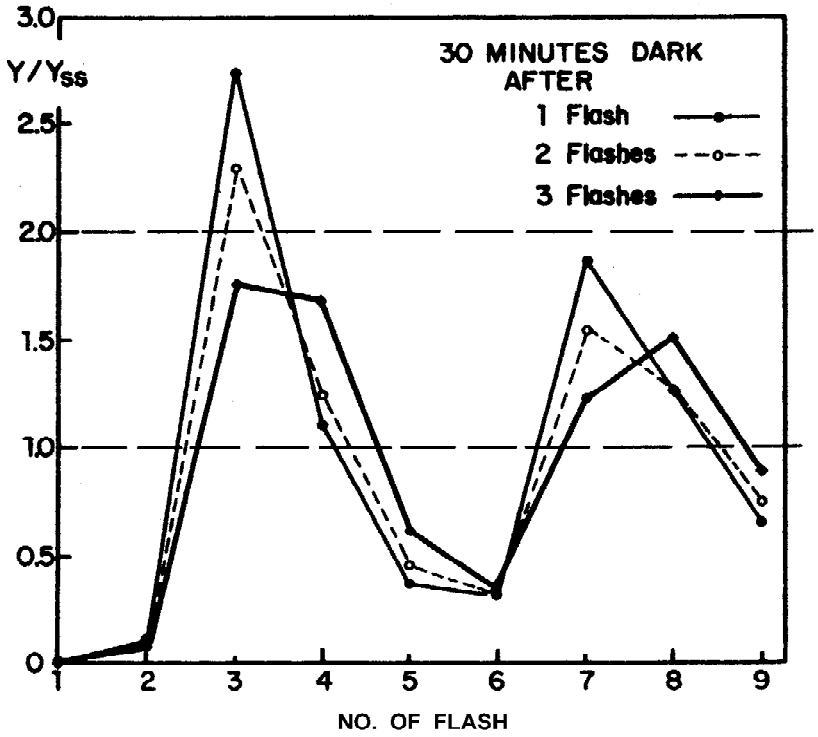

Figure 5. Sequence of flash yield of oxygen (Y/Yss, i.e., normalized to steady-state yields) observed after 30 min darkness following 1, 2 or 3 flashes. Pretreatment: 15 flashes spaced 1 s apart, 5 min dark. Reproduced from Kok et al. (1970).

donor. This model predicts that a maximum amount of oxygen is not evolved on the fourth flash of a series, but on the third one. In this model, three charges are stored in the reaction center after illumination by three flashes. To explain the damping of the oscillation, it was assumed that the probability of commutation between the two Z donors was only ~90%. In this hypothesis, the periodicity of four for oxygen oscillation is not affected by the damping parameter. This model takes into account the experiment performed in weak modulated-light after preillumination by 0, 1, or 2 flashes (Figure 4). In the same paper, the lifetime of the states that had accumulated 1 or 2 positive charges was also measured: ~ 4 s and ~ 30 s, respectively, in the case of *Chlorella* cells *in vivo*.

In 1970, Bessel Kok, B. Forbush and M. McGloin published the classical paper in which the four step charge-accumulation model was proposed

$$S_0 \rightarrow S_1{}^{1+} \rightarrow S_2{}^{2+} \rightarrow S_3{}^{3+} \rightarrow S_4{}^{4+} \rightarrow S_0 + O_2, \quad (1)$$

where S represents a redox state of the oxygen evolving complex, and the superscript represents the positive charge on each complex. A light reaction is required for each transition except when oxygen is released on the last step.

The beauty of this model, purely formally, is that it excludes all unnecessary or unproved hypotheses. The journal *Photosynthesis Research* honored it 25 years after its discovery through a special issue (Govindjee and Renger 1993; Renger and Govindjee 1993). The main conclusion of the above model is that no diffusion of high-energy intermediates is involved in the process of oxygen evolution. In the line of Gaffron and Wohl hypothesis, all the processes, from the absorption of a photon by Chl to the oxygen formation, occur in quasi solid-state system and do not involve any diffusion of molecules. In order to explain that the maximum flash yield appears on the third flash, Kok et al. (1970) assumed that, in dark-adapted material, a major fraction of the centers is already in the S_1 state. At variance with the model of Joliot, a stable positive charge is stored in the dark-adapted reaction center. The experiments were performed either in flashing or in modulated light, with the amperometric method brought to Kok's lab in Baltimore (MD) a few years earlier by P. Joliot from Paris (France). According to the model of Kok, two processes are involved in the damping of the oscillations: 1) about

Figure 6. Top (left to right): the late Robert Hill (discoverer of the Hill reaction; see Walker 2002); the late A. Stanley Holt (authority on chlorophyll chemistry); the late Robert Emerson (see text); and the late Martin Kamen (discoverer of ^{14}C). Photo taken in the early 1950s in Emerson's laboratory in Urbana, Illinois, USA. *Bottom*: the late Eugene Rabinowitch (see text) and the late C. Stacy French (authority on spectroscopy of photosynthetic pigments *in vivo*). Date and photographer, unknown. The photographs are of poor quality, but are reproduced here for their historical value. Courtesy of Govindjee.

Figure 7. A photograph of the author (PJ) with Anne Joliot on the terrace of the Institut de Biologie Physico-Chimique in Paris (June 2002; photograph by W. Majeran).

10% of the centers do not undergo the transition $S_n \rightarrow S_{(n+1)}$ (misses); 2) another fraction of the centers undergoes a double photoreaction, leading to a transition $S_n \rightarrow S_{(n+2)}$ (double hits). The probability of double hits is an increasing function of the duration of the flash. In the case of Joliot et al. experiments, in which flashes of longer duration were used, the probabilities of double hits and misses were coincidentally about equal and the periodicity of the oscillations was close to four. On the contrary, in Kok et al. experiments, the oscillation period is larger than four owing to an excess of misses as compared to double hits. A conclusive argument in favor of Kok et al. model is shown in Figure 5. When chloroplasts are preilluminated by 1 flash and then dark-adapted for 30 min, the oxygen yield on the third flash is about 3 times larger than that measured in steady-state conditions. According to the model of Kok and coworkers, the flash of preillumination followed by a dark period places most of the centers in the S_1 state. This experiment definitely rules out the model of Joliot et al., in which the oxygen yield per flash can not exceed twice that measured under steady-state conditions. Other 2-charge or 4-charge accumulation models able to explain the then available experimental data were proposed by Ted Mar and Govindjee (1972), but it is Kok's model that was definitively accepted and confirmed by number of independent experiments (see Joliot and Kok 1975).

A short time before the publication of Kok et al. (1970) paper, G. Barbieri, R. Delosme and P. Joliot (1969) observed that when *Chlorella* cells were submitted to a series of flashes, the intensity of the luminescence emission (see a review by Lavorel 1975) in the 0.2 s to 10 s time-range oscillates with a periodicity of four (see Delosme and Joliot 2002). The maximum emission is observed on the second flash of the series. In the model of Kok, this implies that, in this time-range, most of the luminescence emission is formed by the back reaction $S_3\text{-}Q_A^- \rightarrow S_2\text{-}Q_A$. In a shorter time-range (<1 ms), Kenneth Zankel (1971) observed a maximum emission of luminescence on the third flash, in phase with the oxygen emission, which can be ascribed to the back reaction $S_4\text{-}Q_A^- \rightarrow S_3\text{-}Q_A$, in competition with the process leading to oxygen formation.

In the same decade, the work of George Cheniae and Iris Martin opened a new area in the understanding of the mechanism of oxygen evolution by giving a molecular support to the formal interpretation arising from the work of Joliot and Kok. Although a possible implication of manganese in photosynthesis was recurrently proposed in the literature, Cheniae and Martin (1969, 1970), both working in Bessel Kok's laboratory, were the first to present a quantitative analysis showing a clear dependence of the yield of oxygen evolution on the manganese (Mn) content in algae and chloroplasts. They established that the restoration of the photosynthetic activity by adding Mn^{2+} to manganese-depleted algae or chloroplasts is strictly light dependent. Number of chemical and/ or physical agents are able to inactivate O_2-evolving centers by removing the manganese without affecting other parts of the photosynthetic apparatus (Cheniae and Martin 1970, 1971). Thus, the work of Cheniae and Martin unequivocally established the involvement of several Mn atoms in the process of water splitting and oxygen evolution. (See Myers, 1987, for a tribute to Kok, and Frasch and Sayre, 2002 for a tribute to Cheniae.)

From the 1970s, the progress in the conceptual understanding of the mechanism of water splitting and oxygen evolution and the photoactivation of the oxygen evolving system opened the possibility to approach this key problem at a molecular level (see Renger, this issue).

Figure 6 shows photographs of Robert Emerson (top) and Eugene Rabinowitch (bottom) (with other researchers of their time). A photograph of Bessel Kok is in the paper by Myers (2002). A recent photograph of myself with Anne, my constant companion in life and science, is shown in Figure 7.

Acknowledgments

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References

Barbieri G, Delosme R and Joliot P (1970) Comparaison entre l'émission d'oxygène et l'émission de luminescence à la suite d'une série d'éclairs saturants. Photochem Photobiol 12: 197–206

Cheniae GM and Martin IF (1969) Photoreactivation of manganese catalyst in photosynthetic oxygen evolution. Plant Physiol 44: 351–360

Cheniae GM and Martin IF (1970) Sites of function of manganese within Photosystem II. Roles in O_2 evolution and system II. Biochim Biophys Acta 197: 219–239

Cheniae GM and Martin IF (1971) Effects of hydroxylamine on Photosystem II. I. Factors affecting the decay of O_2 evolution. Plant Physiol 47: 568–575

Delosme R and Joliot P (2002) Period four oscillations in chlorophyll a fluorescence. Photosynth Res 73: 165–168

Duysens LNM (1952) Transfer of excitation energy in photosynthesis. Thesis, Utrecht University, Utrecht, The Netherlands

Emerson R and Arnold W (1932a) A separation of the reactions in photosynthesis by means of intermittent light. J Gen Physiol 15: 391–420

Emerson R and Arnold W (1932b) The photochemical reaction in photosynthesis. J Gen Physiol 16: 191–205

Förster Th (1948) Intermolecular energy transference and fluorescence. Ann Physik 2: 55–75 [in German]

Franck J and Herzfeld KF (1941) Contribution to a theory of photosynthesis. J Phys Chem 45: 978–1025

Franck J, Pringsheim P and Lad D (1945) Oxygen production by anaerobic photosynthesis of algae measured by a new micromethod. Arch Biochem 7: 103–142

Frasch WD and Sayre RT (2002) Remembering George Cheniae, who never compromised his high standards of science. Photosynth Res 70: 245–247

Gaffron H and Wohl K (1936) Zur Theorie der Assimilation. Naturwissenschaften 24: 81–90

Govindjee (2000) Milestones in photosynthesis research. In: Younis M, Pathre U and Mohanty P (eds) Probing Photosynthesis, pp 9–39. Taylor & Francis, New York

Govindjee and Renger G (1993) In appreciation of Bessel Kok. Photosynth Res 38: 211–213

Govindjee, Beatty JT and Gest H (2003) Celebrating the millennium – historical highlights of photosynthesis research, Part 2. Photosynth Res 76: 1–11 (this issue)

Homann PH (2003) Hydrogen metabolism of green algae: discovery and early research – a tribute to Hans Gaffron and his coworkers. Photosynth Res 76: 93–103 (this issue)

Joliot P (1956) Dispositif ampérométrique de mesure de la photosynthèse. CR Acad Sci Paris 243: 677–680

Joliot P (1961a) Cinétique d'induction de la photosynthèse chez *Chlorella pyrenoidosa.* I. Etude des phases transitoires de la photosynthèse consécutives à une variation d'éclairement. J Chim Phys 570–583

Joliot P (1961b) Cinétique d'induction de la photosynthèse chez *Chlorella pyrenoidosa.* II. Cinétique d'émission d'oxygène et fluorescence pendant la phase d'illumination. J Chim Phys 584–595

Joliot P (1965) Cinétiques des réactions liées à l'émission d'oxygène photosynthétique. Biochim Biophys Acta 102: 116–134

Joliot P (1968) Kinetic studies of Photosystem II in photosynthesis. Photochem Photobiol 8: 451–463

Joliot P (1996) René Wurmser. Photosynth Res 48: 321–323

Joliot P and Kok B (1975) Oxygen evolution in photosynthesis. In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 387–412. Academic Press, New York

Joliot P, Barbieri G and Chabaud R (1969) Un nouveau modèle des centres photochimiques du système II. Photochem Photobiol 10: 309–331

Kok B, Forbusch B and McGloin M (1970) Cooperation of charges in photosynthetic O_2 evolution. Photochem Photobiol 11: 457–475

Lavorel J (1975) Luminescence. In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 225–317. Academic Press, New York

Mar T and Govindjee (1972) Kinetic models of oxygen evolution in photosynthesis. J Theor Biol 36: 427–446

Myers J (1994) The 1932 experiments. Photosynth Res 40: 303–310

Myers J (2002) In one era and out the other. Photosynth Res 73: 21–28

Myers J (1987) Bessel Kok (November 7, 1918–April 27, 1979). Biographical Memoirs (The National Academy Press, Washington, DC) 57: 124–148

Perrin F (1932) Théorie des transferts d'activation entre molécules de même espèce. Cas des solutions fluorescentes. Ann Phys 17: 283–314

Priestley J (1772) Observations on different kinds of air. J Phil Trans R Soc London 62: 147–264

Rabinowitch E (1945) Photosynthesis and Related Processes. Vol I. Wiley (Interscience), New York

Rabinowitch E (1951) Photosynthesis and Related Processes. Vol II, Part I. Wiley (Interscience), New York

Rabinowitch E (1956) Photosynthesis and Related Processes. Vol II, Part II. Kinetics of Photosynthesis (continued); Addenda to Vol I and Vol II, Part I. Wiley (Interscience), New York

Renger G and Govindjee (eds) (1993) How plants and cyanobacteria make oxygen: 25 years of period 4 oscillations. Photosynth Res 38: 211–468

Walker DA (2002) 'And whose bright presence' – an appreciation of Robert Hill and his reaction. Photosynth Res 73: 51–54

Walker DA (2003) Chloroplasts in envelopes: CO_2 fixation by fully functional intact chloroplasts. Photosynth Res 76: 319–327 (this issue)

Whittingham CP and Bishop PM (1961) Thermal reactions between two light reactions in photosynthesis. Nature 192: 426–427

Zankel KL (1971) Rapid delayed luminescence from chloroplasts: Kinetics analysis of components: the relationship to the O_2 evolving system. Biochim Biophys Acta 295: 373–385