Acta Physica Hungarica 55 (1—4), pp. 175—184 (1984)

PULSED POLAROGRAPHIC STUDIES OF PHOTOSYNTHETIC OXYGEN EVOLUTION*

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Synchronously grown cultures of dark-adapted Chlorella fusca cells were illuminated with single flashes and series of flashes from a xenon discharge tube and a nitrogen laser, and the photosynthetic oxygen evolution was measured polarographically under the condition of photosynthetic saturation at ambient temperature. The mechanical and electrical construction of the Joliot-type oxygen polarograph is described. Kinetic analysis of the amperometric signal obtained after single flashes revealed that the diffusion of the oxygen molecules through the thylakoid membrane is the controlling process of the response of the electrode and leads to a lag phase at the beginning of the rise of the signal. The oscillation of the oxygen evolution produced by series of flashes is damped much more with laser flashes. This is explained in terms of the side carrier model of the losses in the oxygen production, assuming that photoactivation of the side carrier by visible light leads to less damped oscillations with xenon flashes. These experiments indirectly corroborate the validity of the side carrier model.

1. Introduction

Photosynthetic oxygen evolution is the most important photochemical reaction on the Earth. For more than 200 years it has been known that higher plants and algae evolve molecular oxygen at the expense of solar energy and water. The release of oxygen forms part of the photosynthetic reaction-chain starting with the absorption of light to the conversion of CO_2 into organic compounds. The substrate from which oxygen is liberated has become a subject of great controversy. It is now generally accepted that the oxygen originates from water either directly (Radmer and Cheniae [1]) or indirectly (Metzner [2]), in accordance with the following photochemical reaction:

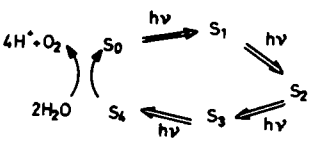
$$2H_2O \xrightarrow{4h\nu} O_2 + 4H^+ + 4e^- . \tag{1}$$

Throughout the history of the Earth, photosynthetic oxygen has established and preserved the oxidizing character of the atmosphere. Protons (H^+) and electrons (e^-) enter mutually coupled series of protolytic and redox reactions, leading to an energization of the thylakoid membrane, to the establishment of conditions for

* In honour of Prof. I. Kovács on the occasion of his 70th birthday and in remembrance of his "Silver Guitar"

photophosphorylation and to the reduction of the terminal electron acceptor, nicotinamide adenine dinucleotide phosphate, thereby inducing the Calvin cycle through which carbon is fixed in the form of carbohydrates and other organic molecules. Though it has become obvious that the photooxidation of water plays a central role as an electron and proton donor in photosynthesis, its mechanism is not known.

In 1968 a very important effect was discovered by Joliot et al [3]: dark-adapted photosynthetic material under series of short (a few microseconds) and saturating light flashes showed a damped oscillatory pattern of oxygen evolution with a periodicity of 4; maximum evolution occurred under the third flash. This clearly demonstrates that oxygen cannot be evolved by direct photolysis of water, but the accumulation of four oxidizing equivalents is needed in the oxygen-evolving system. If the oxidized states of the water-splitting enzyme are denoted by $S_i (i = 0, 1, 2, 3, \text{and } 4)$, the following cycle in oxygen evolution was assumed by Kok et al [4]:



(2)

The basic properties of this "*S*-state" scheme have been well documented (see e.g. Velthuys [5]) and it has become generally accepted as the "Kok model". This has had a great influence on the development of theories explaining finer effects in the oxygen evolution mechanism (Lavorel [6]—[8], Thibault [9], Jursinic [10] and Beckwith and Jursinic [11]) and on the efforts to explore this key photochemical process at a molecular level (Renger [12], Wydrzynski and Sauer [13], Barber et al [14] and Lavorel and Seibert [15]).

One of the methods most frequently used in studying the oxygen evolving apparatus nowadays is pulsed polarography. In the present paper, we discuss the kinetic behaviour of the amperometric signal and stress its importance both in different measuring methods (e.g. in phase polarography) and in the determination of the location of the water-splitting enzyme relative to the thylakoid membrane. Additionally, we present experimental evidence in favour of the side carrier model proposed by Lavorel and Lemasson [16].

2. Experimental

The unicellular green alga Chlorella fusca (from the Algensammlung of the University of Göttingen) was grown under standardized conditions: temperature 24 °C, light intensity $5\,\mathrm{W\,m^{-2}}$. Air enriched in CO_2 up to 5% was bubbled through synchronously-growing cultures with alternating light (14 h) and dark (10 h) periods. The synchronization was checked microscopically. The alga was used for experimentation 3 days after inoculation at a chlorophyll concentration of 0.2 mg/ml.

The polarographic cell (Fig. 1) is in a device similar to that described by Joliot et al [17]. The alga is in direct contact with the platinum electrode (as a monolayer) if a suitable cell concentration ($4.5 \cdot 10^8$ cells/ml) is chosen (taking into account the depth of the cavity (0.15 mm)). The suspension is covered by a light-transmitting cellophane membrane. The chamber between the membrane and the quartz window is filled with culture medium enriched with 0.1 M KCl to ensure the sufficient electrical conductance of the liquid. The steady-state oxygen concentration in the chamber in darkness is determined by the equilibrium between the oxygen uptake by the alga (respiration) and the oxygen supply by the medium, the flow of which is maintained by gravity. Under constant external conditions (temperature, flow rate of liquid, darkness), the steady-state oxygen concentration is reached within several minutes.

The wiring scheme of the system for oxygen measurements consists of three main parts (Fig. 2). The first polarizes the platinum electrode at a voltage of -0.7 V with

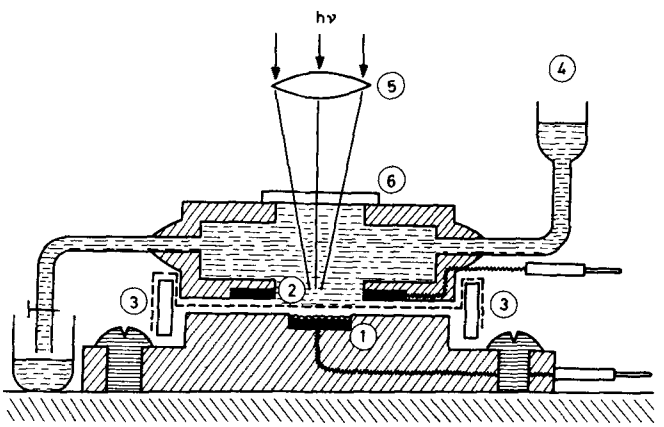


Fig. 1. Polarographic cell for measuring oxygen exchange. 1. Platinum electrode covered with monolayer of algal cells, 2. Ag/AgCl reference electrode, 3. semipermeable membrane (cellophane) fixed by O ring, 4. culture medium flowing through the polarographic cell by gravity, 5. lens focusing the exciting flashes on the Pt electrode, 6. quartz window

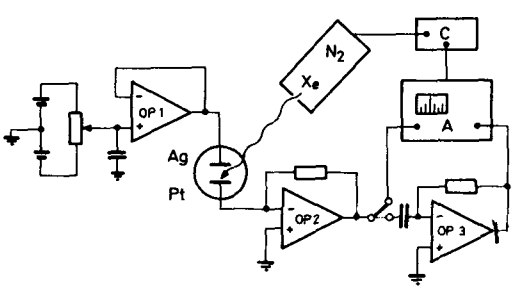


Fig. 2. Simplified wiring scheme of the device for oxygen measurements. OP 1, 2 and 3 operational amplifiers (741, AD 533 and 741, respectively), Ag-Pt polarographic cell, Xe and N_2 light sources (Stroboslave 1539A, General Radio, and Lambda Physik 600 k, respectively), A multichannel analyser (ICA 70), C control unit

respect to the reference Ag/AgCl electrode. This voltage can be adjusted by a battery (to avoid mains noise) and by an operational amplifier (OP 1) with total feedback (to reduce the resistance of the circuit). The second part is a current-to-voltage converter with very low input impedance (OP 2) to keep the time constant of the circuit at a small value. The third part serves the further steps of signal processing. The voltage at the output of the current-to-voltage converter can be fed either directly or via a differential amplifier (OP 3) to the multichannel analyser (A). Rapid changes in the oxygen concentration due to a series of flash illuminations of the alga with a N_2 laser or a Xe discharge tube could be measured in different ways: a) by time-integration of the amperometric signal, b) sampling the signal maximum and the baseline before the flash and calculating the difference, c) subtracting the D. C. component and magnifying the jump of the signal with a differential amplifier. The last method is not so correct as the others because the capacitor causes a flash-dependent baseline shift; thus, the relative amounts of oxygen liberated by flashes can be determined from the amplitudes of the differentiated amperometric signal only with a large experimental error. A control unit (C) provides the synchronization of the device and permits production of different light regimes.

The rates (or the relative magnitudes) of the processes involved in the photosynthetic oxygen evolution can be concluded from the analysis of the kinetics of the amperometric signal caused by a single and saturating flash if the flash duration is much shorter than the smallest reaction time. The duration of the Xe flash (General Radio, Stroboslave 1539 A) is about 3 μs at half-height; 70% of the energy is emitted in less than 25 μs. The duration of the laser flash (N_2 laser, Lambda Physik, 600 k, 337 nm, 1 mJ/cm^2) is even less: 3 ns.

3. Results

The amperometric signals triggered by the flashes under steady-state conditions (repetition rate of flashes 1 Hz) had rise times of 5—15 ms and a half-width of about 40 ms. A typical curve is shown in Fig. 3. The rise time for a definite sample was well defined (with an accuracy of ± 0.5 ms) but changes could be observed in the peak position (5—15 ms) in different branches of the alga. The time courses of the amperometric signals after 2, 3 and 4 flashes applied to dark-adapted alga were identical to the curves obtained with a steady light regime (Fig. 3) (except for constant factors). After the first flash, however, the signal is principally due to a photoelectric artifact caused by illumination of the Pt electrode. Its sign is negative (virtual oxygen consumption) and its amplitude is at least 10 times less than that of the amperometric signal in the steady-state regime. A similar observation was described by Joliot and Joliot [18] with chloroplasts. Although the exact determination of the starting phase of the amperometric signal (less than 2—3 ms) is impossible due to the large negative photoelectric artifact, a lag period can be concluded from the definite position of the flash ($t=0$) and from the shape of the rise in the signal.

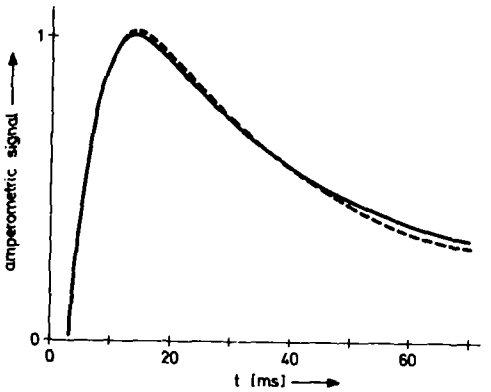


Fig. 3. Oxygen flash yield as a function of flash number n following 3 min dark adaptation. The oxygen yield of any flash, Y_n, is normalized by the steady-state yield, Y_{ss}, reached after many flashes. The sample was Eq. (4) with $\tau = 2$ ms and $\tau^* = 10$ ms (dashed line)

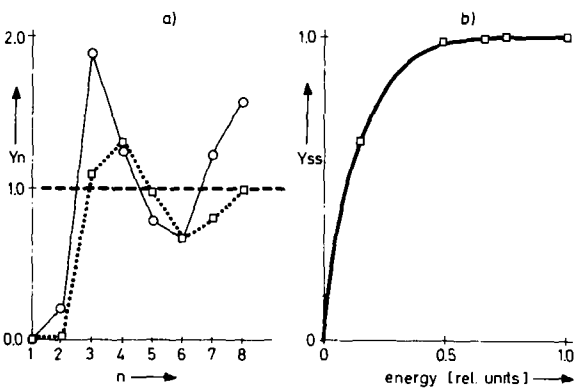


Fig. 4a. Oxygen flash yield as a function of flash number n following 3 min dark adaptation. The oxygen yield of any flash, Y_n, is normalized by the steady-state yield, Y_{ss}, reached after many flashes. The sample was synchronously grown Chlorella fusca at the 10th dark hour, receiving excitation at a rate of 3 Hz from a Xe flash (○——○) or a N_2 laser (□ . . . □)

Fig. 4b. Saturation curve for steady-state oxygen evolution from N_2 laser flashes. The maximum flash energy corresponds to 0.5 mJ/cm^2

Figure 4a presents the oxygen emission sequence upon illumination of dark-adapted Chlorella with a series of saturating Xe and N_2 laser flashes. Y_n is the amount of oxygen evolved by the n-th flash of the series and Y_{ss} is the steady-state value of the oxygen emission. The alga was taken at the end of the cell cycle (10th hour dark) when the oscillation in the oxygen sequence is the most pronounced (Mende et al [19]). The Y_{ss} values for Xe flash and N_2 laser excitation are practically identical, but the pattern is more damped in the latter case (N_2 laser). The first maximum is shifted even from the

third to the fourth flash. If the two flashes are fired simultaneously ($N_2 + Xe$ at $t = 0$), the flash-yield pattern does not differ from that obtained with a single Xe flash excitation. In order to be sure that a saturating N_2 laser flash was applied, the laser pulse was attenuated by stainless-steel screens and the stationary oxygen production was measured (Fig. 4b). It is obvious that the condition for saturated oxygen liberation was largely fulfilled at the highest available laser energy (0.5 mJ/cm^2).

In the double flash experiment, which was carried out similarly as was done by Lavorel [20], the N_2 laser flash was followed by the Xe flash and the steady-state oxygen yield Y_{ss} was measured as a function of the time Δt between the two flashes (Fig. 5). When the dark time between the two flashes was increased, Y_{ss} increased in a biphasic manner, with half-rise times of 300 μs and 1.5 ms, and damped out the oscillation in a parallel manner (not shown). The inset demonstrates the very beginning of the rise, which is clearly not exponential but rather sigmoidal.

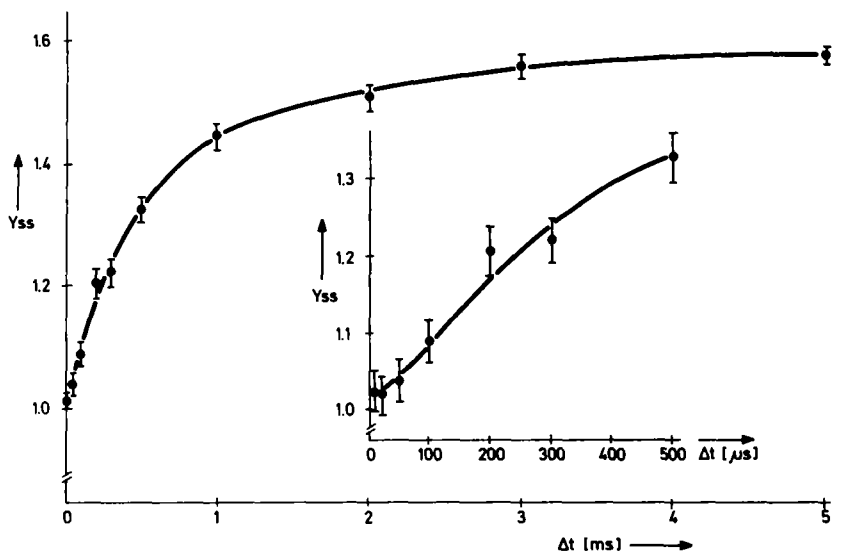


Fig. 5. Steady-state oxygen evolution (Y_{ss}) induced by flashes from N_2 laser and Xe tube in pairs separated by Δt. Inset: The same on an extended time scale

4. Discussion

4.1. Kinetic description of the amperometric signal evoked by a single flash

Several processes may be involved in the time course of the amperometric signal with a maximum at 5—15 ms (Fig. 3): a) the diffusion of oxygen molecules from the cells to the Pt electrode, b) limiting dark reactions in the S state conversion, and c) an electrical artifact due to the time constant of the polarograph. The last two possibilities

could be excluded on the basis of the literature and our own results. Relevant reactions (e.g. proton release) in the S cycle (especially $S_3 \Rightarrow S_4 \rightarrow S_0$) are all faster than $(1\ \text{ms})^{-1}$ (Junge [21]). This means that the oxygen should be evolved within 1 ms after the flash. Our signal maximum cannot be ascribed to an electrical artifact, because the peak position could be changed by experimental modifications not related to the electrical time constant of the polarograph (e.g. different branches of the alga). The first assumption remains: the time course is related to the diffusion of the oxygen molecule from the water-splitting enzyme to the Pt electrode [17].

An attempt to describe the time course of the signal in terms of diffusion leads to complications, revealing that the problem cannot be treated as simple diffusion over a definite distance. The time (τ) needed to cover a distance (x_0) by diffusion is

$$\tau = \frac{x_0^2}{4D}, \tag{3}$$

where D is the diffusion constant. In our case $x_0 \cong 4\ \mu\text{m}$ (taking into account the average microscopically measured sizes of the alga) and $D = 2 \cdot 10^{-5}\ \text{cm}^2\ \text{s}^{-1}$ (diffusion constant of the oxygen molecule in water [17]). This leads to $\tau = 2\ \text{ms}$, a time considerably less than the experimentally measured value.

In order to explain the time difference, a delaying effect must be found in the oxygen-release mechanism of the alga. The process might be visualized as follows. The water-splitting enzyme is situated within the chloroplast thylakoid membrane, having access to the aqueous phase on the inner or lumenal side. The oxygen molecule evolved in the inner phase should penetrate the membrane in order to reach the electrode. The diffusion through the thylakoid (which cannot be observed by proton detection with pH-sensitive dyes) is responsible for the delay of the amperometric signal.

In a model of the processes, the initial Dirac δ oxygen pulse flows away in two layers with different diffusion constants: D^* in the membrane (thickness x_0^*) and D between the outer side of the membrane and the Pt electrode (distance x_0). The amperometric signal ($i(t)$) is proportional to the number of oxygen molecules reaching the Pt electrode in the time between t and $t+dt$. The differential equation of the diffusion (Fick's second law) should be solved for a definite initial condition (the oxygen distribution is $\delta(x)$ at $t=0$) and for a definite boundary condition (at $x=x_0$ the distribution function can be calculated from the Fick law without boundary conditions, see e.g. [22]). The solution can be obtained in integral form by expressing the method of convolution applied to solve the problem:

$$i(t) = \text{const} \int_0^t [\vartheta \cdot (t-\vartheta)]^{-3/2} \cdot \exp\left\{-\frac{\tau^*}{\vartheta} - \frac{\tau}{t-\vartheta}\right\} d\vartheta, \tag{4}$$

where τ and τ^* are the average diffusion times through the layers and are defined according to Eq. (3).

Different τ^* values were chosen for the measured τ value and the integral in Eq. (4) was calculated numerically by computer. The best fit with the measured curve

was obtained with $\tau^* = 10$ ms (Fig. 3), the measured and calculated curves then coinciding within experimental error.

Further support for the validity of our explanation is the true description of the lag phase at the beginning of the rise of the measured curve. The sigmoidal rise is a consequence of the differences in the diffusion times in the series-coupled diffusion problem. The time of diffusion through the thylakoid membrane depends on many factors, including the physiological state of the membrane. This leads to different half-rise times in the amperometric signal in different branches of the alga.

The knowledge of the actual diffusion time may be very important in some kinetic methods. In particular, care must be taken with the modulated oxygen electrode (see e.g. Sinclair et al [23]) to avoid diffusion being the rate-limiting step in the examined reactions.

4.2. Characteristics of the oxygen-yield pattern evoked by a series of N_2 laser flashes

Our experimental results in Fig. 4 clearly indicate the peculiar effect of N_2 laser flashes on the oxygen sequence. This has already been noted by Weiss et al [24], who found that the pattern of flash yields was very similar to that produced by the attenuated Xe flash. However, they were unable to obtain a complete saturation curve and assumed that the oxygen evolution system was somehow inactivated by the ultraviolet laser flashes. Below we attempt to obtain a clearer insight into this problem. The oscillation of oxygen evolution is damped more strongly by N_2 laser flashes than by Xe flashes, although both flashes could saturate the steady-state oxygen production. This indicates the appearance of loss mechanism(s) in the oxygen-evolving apparatus. Kok et al [4] distinguished two factors contributing to the damping: miss ($S_i \Rightarrow S_i$) and double hitting ($S_i \Rightarrow S_{i+2}$). The latter is negligible in our case, due to the short duration of the flashes. (It must be noted that, even with very short excitation (5 ns), some double hitting could be observed by Jursinic [25].) The miss factors are usually treated only formally in the Kok model and in other mathematical versions ([6], [9] and [11]); only speculation exists as to the occurrence of misses at a molecular level. The mechanisms leading to losses in oxygen production were recently reviewed by Lavorel [8], [20]. In his classification, losses can occur either in the reaction centre (or in the antenna) or in the water-splitting enzyme. The former type of misses is termed photochemical (photophysical) misses, and the latter conservative misses.

The double flash experiment (Fig. 5) demonstrates that there is no extra photophysical and photochemical miss due to the N_2 laser excitation. Since the flash energy (0.5 mJ/cm^2) is not oversaturating, we do not have to consider non-linear effects causing quenching in the antenna or in the reaction centre. The turnover time of the second photosystem (PS II), determined principally by the reoxidation time of the reduced primary acceptor Q (300 μs) and by the reoxidation time of the plastoquinone pool (1.5 ms), proves the normal action of the reaction centre under N_2 laser excitation

from the point of view of oxygen evolution. The almost identical steady-state oxygen productions evoked by the two types of flashes provide an additional argument for increased conservative misses with N_2 laser excitation. The charges separated in the reaction centres will enhance the degree of oxidation in the water-splitting enzymes by the same overall quantity for both the N_2 laser flash and the Xe flash (the Y_{ss} values are identical), but the S states will be "mixed" differently, causing different damping in the oxygen-yield oscillation. Several models have been constructed to interpret the nature of the conservative misses [3], [4], [7], [16]. The tendency in these theories is towards more cooperativity between the water-splitting enzymes and the different centres. While the Kok model is absolutely non-cooperative, the E—P (enzyme—pigment) model [7] made a radical step towards more cooperativity by considering E kinetically distinct from the reaction centre. An intermediate version is the side carrier model [16], which allows a lateral (possibly free) carrier C to store a + charge reversibly (Fig. 6). The free-diffusing species picks up a charge on S_3 and feeds it back to the system S_0. Unequivocal experimental evidence in favour of any of the cooperative models has not yet been found. Our results with N_2 laser flashes can be interpreted fairly well in the framework of the side carrier model, while the other models can be excluded.

Jursinic [25] observed that the oxygen yield pattern produced by Xe flashes was not modified if dye laser flashes (duration 5 ns, central wavelength 584 nm) replaced the Xe flashes. If this fact is compared with our finding, it can be concluded that the stronger damping is due to spectral reasons and not to the very short pulse duration of the N_2 laser.

In Fig. 6 the redox level of the C/C^+ couple is shown relative to that of the S states. Let us suppose that the side carrier may exist either in low-potential (LP) or in high-potential (HP) forms and that the transition may be triggered by external conditions such as white light (Xe flash) but not near UV excitation. (This is similar to the change in the redox potential of cytochrome-b559 induced by the change from autotrophic to heterotrophic conditions [26].) The low-potential form of C picks up a charge on S_3 with greater probability than does its high-potential form, and thus the

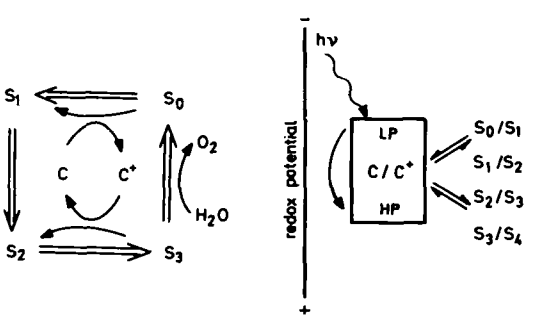


Fig. 6. Side carrier (C) mediating among the differently oxidized states of the water-splitting enzyme (S_i) and their schematic positions on the redox scale. LP and HP are the low and high-potential forms of the side carrier, respectively

number of conservative misses is higher. The stable form is the LP form which can be converted to the HP form by white light absorption, but not by light of 337 nm. The side carrier itself or its sensitizer should have an absorption band in the visible part of the spectrum.

We may conclude that the special action of the N_2 laser flashes on oxygen evolution can be explained in terms of the side carrier model. Photoactivation (absorption) in the visible range seems to be an important photophysical property of the side carrier.

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