An Amperometric Study on the Time Constants of Oxygen Release in Thylakoids of *Nicotiana tabacum* and *Oscillatoria chalybea*

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Measurement of photosynthetic oxygen evolution in tobacco chloroplasts and thylakoids of the filamentous cyanobacterium *Oscillatoria chalybea* by means of the "Three-Electrode-System", described by Schmid and Thibault in 1979, yields half-rise times for the directly measured amperometric O_2-signal of approximately 2 msec for tobacco chloroplasts and slightly more than 2 msec for thylakoids of *Oscillatoria chalybea*. An estimate of the possible contribution of the diffusion time required for oxygen to leave the thylakoid membrane (7.5 nm thickness) or the chloroplast (diameter 4−5 μm) might bring the measured O_2-release time below 1 msec in full agreement with measurements by Joliot *et al.* in 1966.

Introduction

If photosynthetic oxygen evolution is measured as the consequence of short saturating light flashes, the picture of a damped oscillation with a periodicity of four is obtained. The original observation was made many years ago by Joliot and Kok [1, 2]. The up-to-now still valid interpretation for the observation has been given already many years ago by the Kok model [2] demonstrating that four light quanta have to be successively absorbed, leading to the successive accumulation of four positive charges, before water can be split to give molecular oxygen. The attempt to identify intermediates of this water oxidizing reaction or oxygen precursors has failed so far. Mass spectrometric experiment by Radmer and Ollinger [3] or Bader, Thibault and Schmid [4] have shown that apparently up to the S_3-state no defined water molecule that was not exchangeable within the experimental times of minutes was bound to any of the S-states. The constraints of these results are not easily incorporated in thermodynamic considerations on photosynthetic water oxidation which would easier manage with intermediates of the reaction such as peroxidic components [5]. The time required for the charge accumulation has been measured [6] and was ≈ 1.5 ms for the whole "parcours" from S_0 to S_4 or ≈ 1.2 ms for the $S_3 \rightarrow S_4$ transition. It was accepted knowledge that oxygen evolution itself following this charge accumulation was the matter of a few milliseconds [7] until a recent report by Plijter *et al.* [8] who presented experimental data leading to the conclusion that photosynthetic oxygen release following charge accumulation might be a process being $10-100$ times slower than originally thought [7]. In the present paper we present a signal analysis of oxygen release, measured upon illumination with our fast three-electrode system [9] in tobacco chloroplasts and thylakoids of the filamentous cyanobacterium *Oscillatoria chalybea*.

Materials and Methods

Measurements of oxygen evolution as the consequence of short saturating light flashes were carried out with the "Three Electrode-System" described in detail earlier by Schmid and Thibault at room temperature and at pH 7.5 [9]. Signals were digitized and computer-analyzed with an Atari Mega ST 4.

Flashes of a duration of 8 μsec were provided by a Stroboscope (1539 A of General Radio).

Chloroplast preparations of tobacco were prepared according to Homann and Schmid [10] and *thylakoid preparations* of the filamentous cyanobacterium *Oscillatoria chalybea* were made according to the procedure described by Bader *et al.* [11].

Results

Amperometric signal amplitudes due to photosynthetic oxygen evolution measured in chloroplast preparations as the consequence of short saturating light flashes depend on the cathode potential used. Fig. 1 shows the signal amplitudes under the 3rd flash of a train of light flashes spaced either 300 msec or 1 sec apart in dependence on the polarization voltage. It is clearly seen that increasing the polarization voltage from −148 mVolts to −709 mVolts leads first to an increase in signal amplitudes (given in mVolts) until a saturation value will be reached with higher polarization voltages (Fig. 1). The signal amplitude in the case of tobacco depends, due to the life time of the S-states, on the dark time between flashes (Fig. 1 A, B). Dark times of 1 sec between flashes permit partial deactivation and lower the signal amplitudes at any polarization voltage (Fig. 1 B). In the case of *Oscillatoria chalybea* where S-state deactivation is slower, as already reported earlier [12], the dark time of 1 sec between flashes has no consequence yet on the observed signal amplitudes (Fig. 2 A and B). The observed polarogram justifies the general use of a polarization voltage around −600 to −700 mV for oxygen analysis (Fig. 1 C and 2 C). Due to the high polarization usually applied in such measurements, samples find themselves, due to oxygen reduction *i.e.* consumption by the electrode, under fairly anaerobic conditions. After prolonged sedimentation times it

may happen that oxygen-evolution appears inhibited as described by Plijter *et al.* [8]. The three described "all or nothing inhibition (8)" is with our device observed at best with *Oscillatoria* and is then due to the anaerobiosis effect described in detail by Bader *et al.* [4] and Bader and Schmid [13]. If the amperometric oxygen signal of a third flash (flash duration 8 μsec) in a train of saturating flashes is analyzed with tobacco chloroplasts, it is seen that oxygen release starts immediately (Fig. 3). Despite the fact that such a signal usually starts out with an electric parasite the duration of which is ≈ 1 msec (in Fig. 3 exactly 0.95 msec), the overall shape of the signal shows that oxygen is released from the very beginning yielding a total signal rise time of 8.01 msec or a half-rise time of the order of less than 3 msec. The half-width of the signal is 22.87 msec. The same signal analysis for thylakoid preparations of *Oscillatoria chalybea* yields an only slightly slower signal with (as is always the case) an incidentally differently shaped parasite the total duration of which is 1.33 msec (Fig. 4). The total rise time is 11.4 msec and the half rise time being slightly longer then 3 msec. The half-width of the signal is 33.06 msec. The contribution of the parasite to the signal characteristics can be determined by studying their dependence on the concentration of oxygen evolving sources (*i.e.* thylakoids). As we have proportionality between signal amplitude and oxygen concentration (Table I), with the parasite size staying more or less the same (experiments not shown in

Table I. Dependence of the amperometric signal amplitude on the concentration of oxygen-emitting sources measured with stroma-freed tobacco chloroplasts.

Thylakoids corresponding to μg Chlorophyll	Amplitude [mV]	Signal Characteristics		
		Half-rise time [msec]	Rise-time [msec]	Half-width [msec]
100	238	1.47	5.45	24.75
75	191	1.47	5.45	23.91
50	129	1.47	4.83	28.13
37.5	85	1.47	4.83	37.33
25	53	1.68	5.04	178.47

Cathode potential −680 mV. The time characteristics given are values in which the contribution of the electrical parasite signal to the oxygen signal has been eliminated; Tobacco used was: *N. tabacum* var. John William's Broadleaf. Measurements were carried out at room temperature.
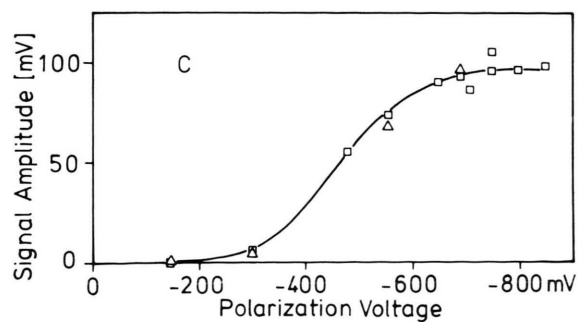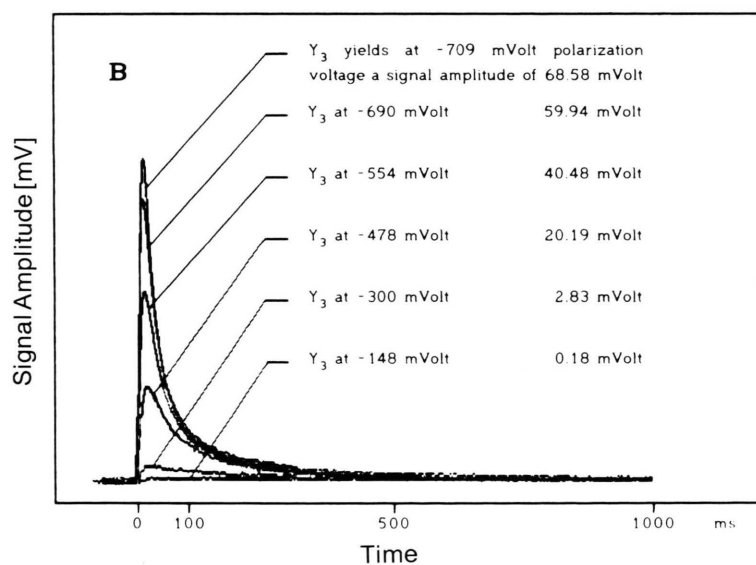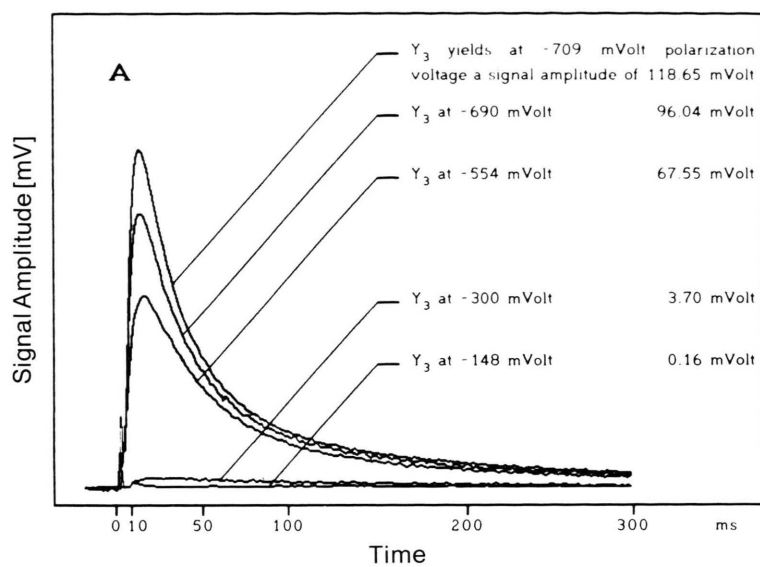
A

Y$_3$ yields at −709 mVolt polarization voltage a signal amplitude of 118.65 mVolt

Y$_3$ at −690 mVolt   96.04 mVolt

Y$_3$ at −554 mVolt   67.55 mVolt

Y$_3$ at −300 mVolt   3.70 mVolt

Y$_3$ at −148 mVolt   0.16 mVolt

B

Y$_3$ yields at −709 mVolt polarization voltage a signal amplitude of 68.58 mVolt

Y$_3$ at −690 mVolt   59.94 mVolt

Y$_3$ at −554 mVolt   40.48 mVolt

Y$_3$ at −478 mVolt   20.19 mVolt

Y$_3$ at −300 mVolt   2.83 mVolt

Y$_3$ at −148 mVolt   0.18 mVolt

C

Fig. 1. Oxygen evolution in chloroplasts of *Nicotiana tabacum* var. John William's Broadleaf measured under the third flash (Y$_3$) of a train of 15 short saturating light flashes. Time course of the signal amplitude in dependence on the cathode potential. Sedimentation of the chloroplasts on the bare electrode 15 min; dark adaptation 15 min; A. dark interval between flashes 300 msec; B. dark interval between flashes 1 sec; C. Polarogram belonging to the conditions of A. (△) are points obtained from the amplitudes shown in Fig. 1 A.
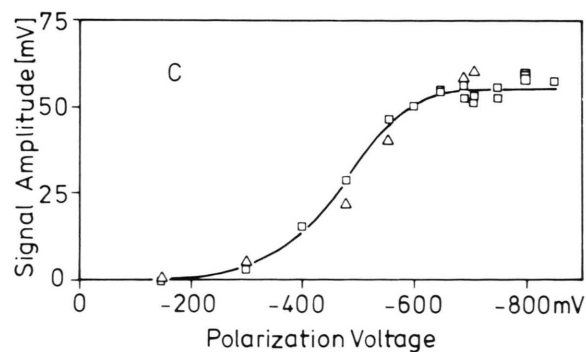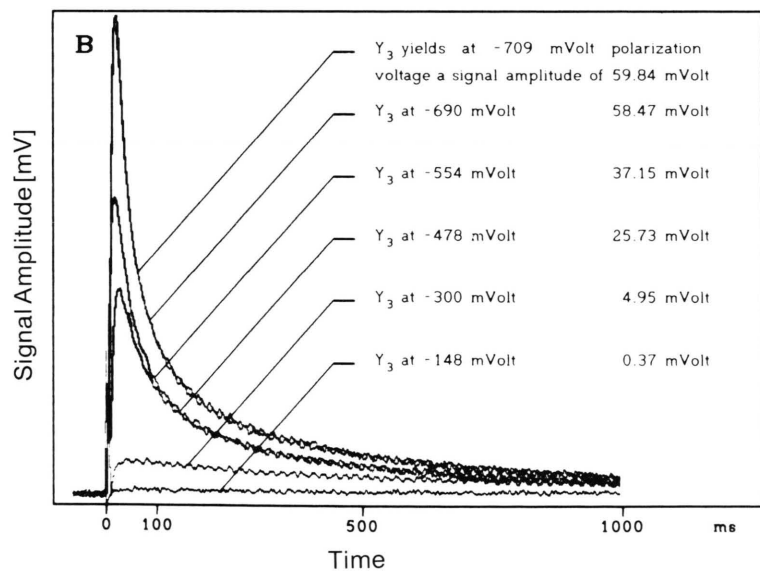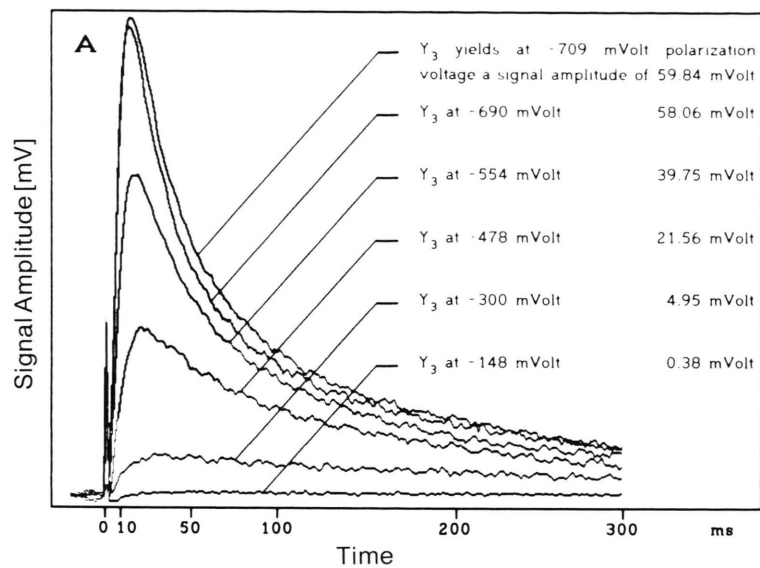
Fig. 2. Oxygen evolution in a thylakoid preparation of the filamentous cyanobacterium *Oscillatoria chalybea* measured under the third flash (Y_3) of a train of 15 short saturating light flashes. Conditions as in Fig. 1. A. dark interval between flashes 300 msec; B. dark interval between flashes 1 sec; C. Polarogram for the conditions of A. ($\triangle$) are points obtained from the signal amplitudes shown in Fig. 2A.

detail, but see Fig. 1 A, 1 C and 2 A, 2 C), contribution of the parasite to differently sized oxygen signals of the type shown in Fig. 3 and 4 permits the correct elimination of the parasite contribution itself. Table I shows that, if the parasite signal is thus excluded from the signal proper in tobacco thylakoids, an identical half-rise time of 1.5 msec is observed for all concentrations used. The rise time of such typical signals shown in Fig. 3 and 4 seems

not much artificially shortened by the interference with an oxygen uptake reaction. This is best demonstrated by the fact that the amplitudes of such signals in a train of short saturating light flashes yield perfect experimental Kok-sequences (Fig. 5 A and B) which, if mathematically analyzed in the four state Kok-model, yield fits with a relative quadratic deviation of less than 1% [11]. In these analysis we have made with the first four
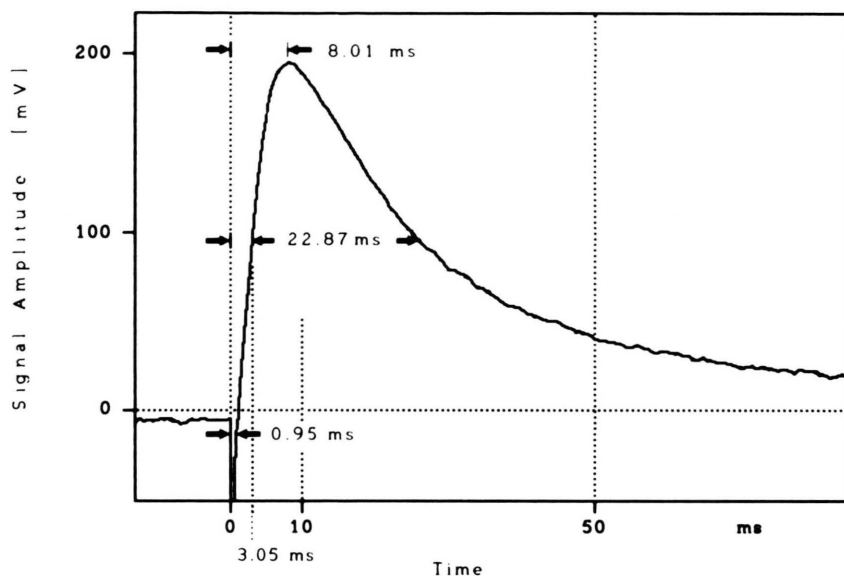

Fig. 3. Signal analysis of the third flash (Y_3) from a sequence measured at -680 mV cathode potential with chloroplasts of *N. tabacum* var. John William's Broadleaf. Sedimentation and dark adaptation before the first flash 30 min; dark time between flashes 300 msec. The tracing gives the time resolution of the signal amplitude in mV. The signal starts out with an electrical parasite of 0.95 msec. The total rise time is 8.01 msec and the half-width of the signal 22.87 msec. The half-rise time (parasite included) is below 3 msec.
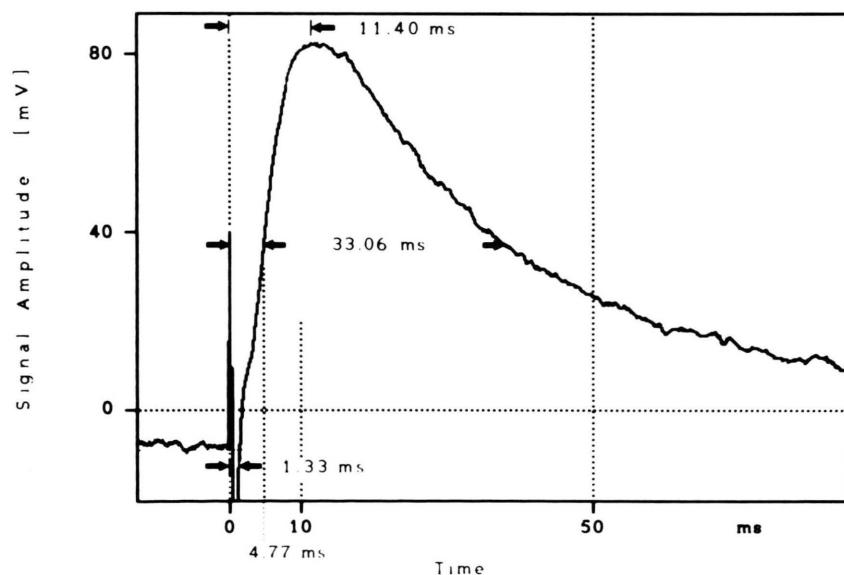
Fig. 4. Signal analysis of the third flash (Y_3) from a sequence measured at -680 mV cathode potential with thylakoids of *Oscillatoria chalybea*. Sedimentation and dark adaptation 15 min. Other conditions are identical to those of Fig. 3. The time resolution of the signal shows a differently shaped parasite of a width of 1.33 msec and a half-rise time of ≈ 3 msec. Total signal rise time and half width of the signal are indicated.
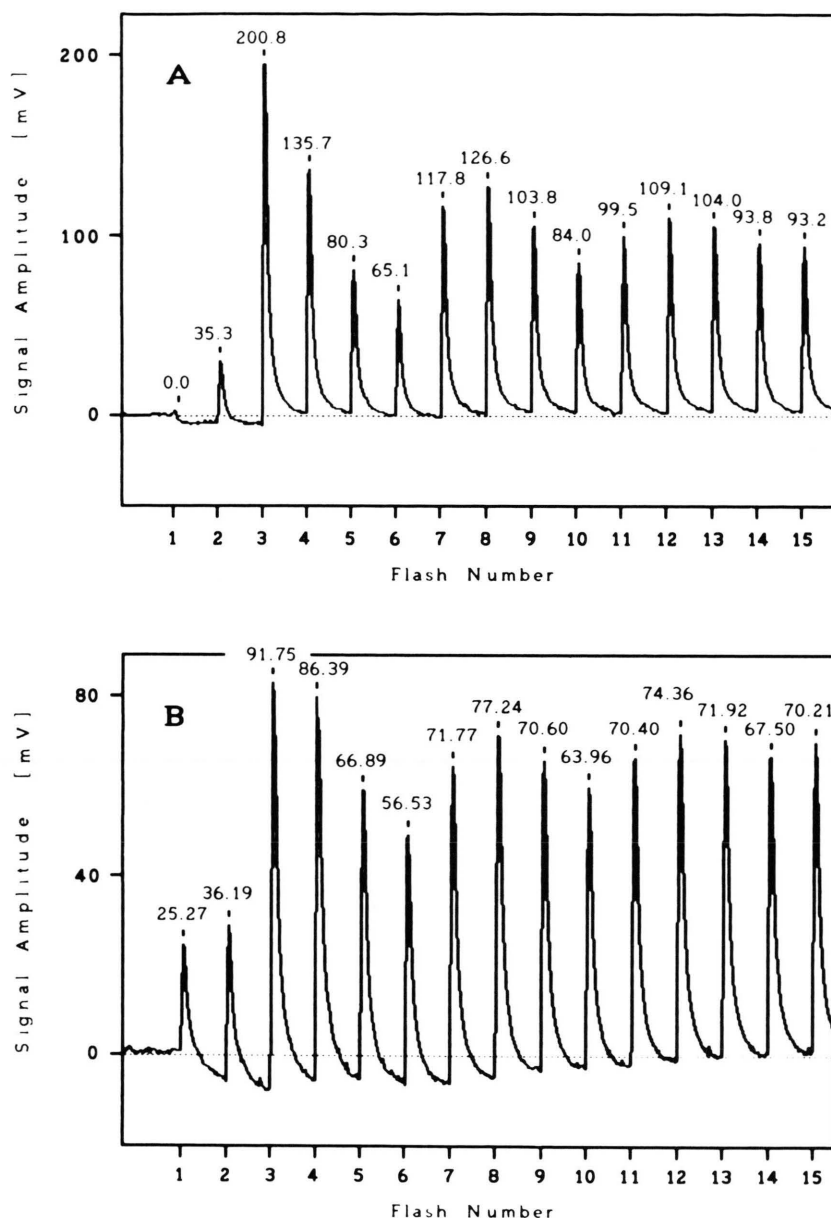
Fig. 5. Direct tracing of the flash pattern belonging to the signals analyzed in Fig. 3 and 4. The numbers on the spikes give signal amplitudes in mVolts. A. *N. tabacum* var. John William's Broadleaf; B. *Oscillatoria chalybea*.

oxygen amplitudes of the sequence a mathematical fit on the "shape factors" which are the three transition probabilities α, β and γ according to the recurrence law established by Lavorel [14]. That oxygen release is the matter of a few milliseconds, as already estimated by Joliot many years ago [7] and as demonstrated by indirect recent evidence [15], may already be seen from the crude time resolution of photosynthetic oxygen release upon the onset of continuous illumination (Fig. 6). Fig. 6A shows the oxygen gush pattern of dark adapted *Oscillatoria chalybea* upon illumination. Time resolution of the onset of oxygen release within the first fast gush (Fig. 6B) shows oxygen evolution already within the first millisecond which in this case is not disturbed by a flash induced electrical parasite.
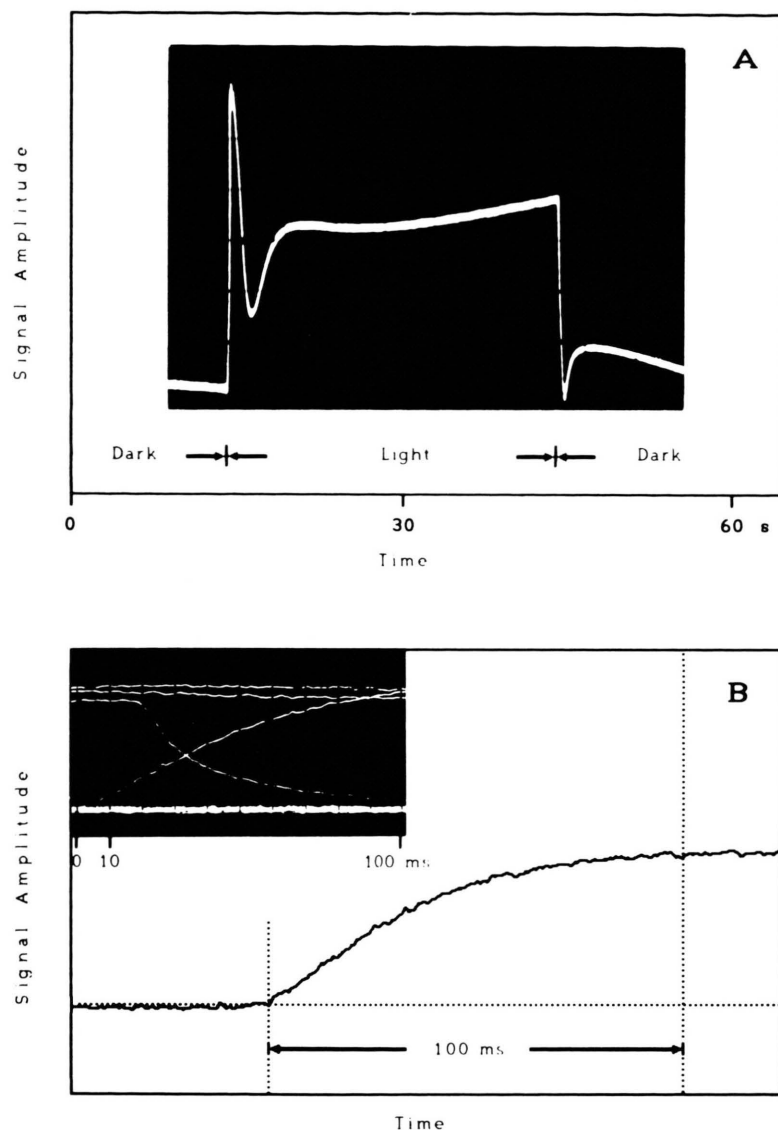
Fig. 6. A. Oxygen gush measured in thylakoids of *Oscillatoria chalybea* during a continuous light illumination of 30 sec. Dark adaptation before the onset of illumination 15 min.
B. Time resolution of the oxygen signal in the first part of the gush pattern. Inset: original experimental recording with the light-on and light-off response.

Discussion

For many years it appeared fully established that the time required for photosynthetic oxygen release, after the ≈ 1.5 msec requiring parcours from $S_0 \rightarrow S_4$, was the matter of a "few milliseconds" [7], meaning that O_2-evolution following the S_4-state was immediate, having besides the diffusion time out of the water-splitting membrane system or the organism *(e.g. Chlorella)* an only very short time requirement. In fact, Joliot *et al.* determined already in 1966 this time to be 0.8 msec

[7]. This was accepted knowledge until a recent report by Plijter *et al.* [8] who proposed half-times required for the release of oxygen of $30-130$ msec. As the implications of this observation with respect to the water-splitting mechanism are manifold and important, we attempted to differentiate between the two observations by the signal analysis of the present paper, carried out with our fast "Three-Electrode-Device" [9]. This requires in principal just the demonstration of how and to what extent the rise kinetics of the amperometric signal

correlate with the oxygen release time. It is beyond the scope and the intention of the paper to present *via* a detailed kinetic analysis, including all possible diffusion situations, the most exact oxygen release time. The intention of the paper is just to discriminate between the order of magnitude of the release time given by Joliot *et al.* [7] and that given by Plijter *et al.* [8]. In this context it should be noted that if we had not published the present study, work by Lavergne [15] has in this spirit already put the final point to this controversial question. Lavergne shows *via* the measurement of absorption changes that in anaerobic algal cells photosynthetically evolved oxygen is intracellularly used for cytochrome *c* oxidation by cytochrome *c* oxidase. An absorption change pattern in dependence on the flash number is obtained which essentially corresponds to the usual Kok-pattern. In these measurements the fastest component (heme a_3) reaches its absorption maximum at around 3.5 msec, implying that photosynthetic oxygen release had to be faster than this [15].

The availability of the Lavergne paper [15] justifies that our argumentation can be simple, if not to say elementary:

The principle of our measurements is that of all amperometric measurements, namely that oxygen evolved by the alga or chloroplast is readily reduced, if an immersed electrode of a sufficiently negative potential is used [9, 16, 17]. In the immediate vicinity of the electrode the consumed oxygen is renewed just by diffusion, if from a certain voltage onward the electrochemical current is only limited by this transfer speed. Working under these conditions is characterized by the fact that the electrochemical current becomes independent on the voltage (plateau of the polarogram) and *the observed current then is proportional to the O_2-concentration* between the electrode and the assay solution. This is the overall principle to use in any amperometric measurement. Interpretation of measurements on the slope of the polarogram is much more complex with respect to diffusion and electrode kinetics. In the present study within the outlined principle we use a three-electrode-device described in detail already 11 years ago [9]. The regulation of the cathode potential (usually -0.6 to -0.7 V) in reference to the usual Ag/AgCl anode is achieved by means of a differential amplifier (Fig. 1 in ref. [9]). In order to maintain the poten-

tial difference zero between the two entrances the amplifier produces a current which opposes itself to that caused by the electrochemical reaction. A 10 kΩ resistance inserted into the circuit of an auxiliary third electrode (Ag or Pt) permits to catch the signal in form of a variable tension which is fed to a recording device. This system is fast in response and sensitive and *permits the simultaneous measurement of O_2-uptake and evolution phenomena* of different kinetics [9]. As described earlier, the electrode set-up represents an open system, fully exposed to oxygen of the ambient atmosphere [9]. A drop, containing the oxygen-evolving or -uptaking sources is put on the electrode surface. These particles sediment on the polished large electrode surface and a flux equilibrium is established in which oxygen diffuses from the ambient atmosphere into the drop, passing by the sedimented particles (on the immediate electrode surface), to the electrode where it is constantly reduced *i.e.* consumed.
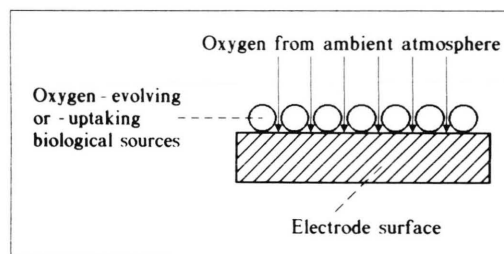
Scheme:
Functional mode of the **open** electrode device

Upon illumination the oxygen-active particle preparation on or at the electrode surface add to this flux if O_2 is evolved and take some away, if they take up O_2. Hence, this device measures the O_2-metabolism of only those individuals which are in direct contact with the electrode surface. The next adjacing layer or farther away laying ones do not contribute at all or not significantly to this fast signal. In this set-up evolution increases the measured tension and uptake diminishes it, hence, evolution and/or uptake are *identified* as such. Needless to say that stirring the assay yields no signal and too dense packing in the sediment ruins the functionality of the device according to what has been said above and described earlier [9]. According to the sketched properties of the device inter-

pretational errors can come from situations in which a positive signal is not produced by O_2-evolution but rather by an inhibited uptake (for example a photoinduced inhibition of O_2-uptake see discussion in [11]). Also the relaxation of our system (the technical and biological one taken together) is fast enough to avoid the "pile-up of pulses" described sometimes when measuring O_2-evolution as the consequence of μsec flashes [18].

A comparison of our measurements with those of Plijter *et al.* shows first that we are able to obtain a conventional polarogram (Fig. 1 C and 2 C) and do not observe a drop of signal amplitudes in the region of polarization voltages of -500 to -750 mV (Fig. 1 B in ref. [8]). We are sure to measure oxygen as is Lavergne [15] as the signal analyzed is the third signal of an amperometric signal series measured as the consequence of short saturating light flashes showing the typical Joliot [1]/ Kok [2] pattern (Fig. 5). In this sense things have become easier since the times of Joliot's measurements in 1966 where assumption-free the prove had to be furnished that the amperometric signal observed was due to oxygen. As we have polarogram, measurements on the plateau of the polarogram yield the strict proportionality between signal amplitude and oxygen concentration (Table I) predicted. *"Oxygen concentration"* here means concentration of oxygen emitting sources *i.e.* photosynthetically active particles which in the experiment shown in Table I are tobacco thylakoids. As the electrode system used is a *bare polished* large surface electrode system on which the sources *i.e.* thylakoids are in intimate contact with the electrode surface, diffusion distances are very short and correspond with a „*stroma-freed chloroplast*" (that is the lamellar system) of a diameter of $4-5$ μm to an average diffusion distance of ≈ 2 μm. With an oxygen diffusion coefficient in an aqueous medium of $D = 2.3 \times 10^{-5}$ cm² sec⁻¹ at room temperature this diffusion distance Δx permits an estimate of the corresponding diffusion time t according to $\Delta x = \sqrt{2 D \times t}$ which would be for ≈ 2 μm at room temperature approx. 1 msec. This is to say that besides diffusion out of the membrane system or the organism there is no appreciable diffusion distance to consider and that diffusion from the source of the used type, emitting oxygen according to its inherent mechanism (with its inherent half-rise time), brings the oxygen

emitted to the electrode surface within one millisecond.

Contribution of diffusion to the observed oxygen release kinetics are certainly lesser if single stranded isolated mucoid-free cyanobacterial thylakoids are used instead of stroma-freed chloroplasts. Thus, we think that the half-rise time measured in Table I is essentially the rise time of the oxygen source plus a constant increment due to diffusion as discussed. Of course, the observed signal amplitude and with it the rise kinetics are to a certain extent influenced by convolution with decay kinetics but within the overall signal shapes shown in Fig. $1-4$ a substantial effect is only to be expected from a fast (msec) decay, obviously not contained at first glance in the decay kinetic shown (half-width of the signal 30 msec) in Fig. $3-4$ or in Fig. 1 C of ref. [8].

Measurements of the onset of oxygen evolution upon flash illumination with our electrode device are complicated by and interfere with an electrical parasite with a duration of ≈ 1 msec. The here presented signal analysis *with* this parasite included shows that in tobacco thylakoids (Fig. 3) our measurements yield directly a half-rise time of the signal between $2-3$ msec, confirming values reported by Sinclair and Arnason [19]. However, experiments of the type shown in Table I permit to determine with fair precision the interference of the parasite contribution to the signal onset, its half-rise time and the half-width of the signal. If the parasite is thus eliminated from the signal proper, a half-rise time of ≈ 1.5 msec is observed for tobacco (Table I), confirming also the skillful experiments by Etienne [20]. At this point we should like to note that we are particularly interested in the implications of Plijter *et al.* [8] observation which would ease the interpretation and explication of a number of our observations made with the filamentous cyanobacterium *Oscillatoria chalybea* [11, 12]. If Plijter's *et al.* observations were true the thermic reaction leading to water-splitting or oxygen evolution is topographically separated from the charge accumulation complex. The statement of these authors [8] that charge accumulation can always procede to the S_3-state with the limiting event being the succeeding O_2-release would make the occurrence of metastable S_3 described in the filamentous cyanobacterium *O. chalybea* ([11] and Fig. 5 B) less surprising. However, our measure-

ments do not support yet half-rise times for oxygen release of $30-130$ msec. Our measurements yield consistently values around $1.5-2$ msec. If the contribution of diffusion out of the membrane system is taken into account photosynthetic oxygen release as measured in this paper might require less than 1 msec (possibly ≈ 0.5 msec in Table I). If one judges the conclusion appropriate that photosynthetic oxygen release itself can only be faster, than what is *directly* seen (as half rise-time of $2-3$ msec (Fig. 3 etc.)) Joliot's original estimate of 0.8 msec made 22 years ago [7] seems to preserve its validity and still seems very close to reality.

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