A Study on Oxygen Evolution and on the S-State Distribution in Thylakoid Preparations of the Filamentous Blue-Green Alga *Oscillatoria chalybea*

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When thylakoid preparations of the filamentous blue-green alga *Oscillatoria chalybea* are exposed to short (2 or 8 µs) saturating light flashes, the oxygen evolution pattern can be distinguished in several respects from the one usually observed in Chlorella. Thus, it appears that a substantial electrochemical signal is already seen under the first flash with maximal flash yield always occurring under the fourth flash. This refers to dark adapted preparations (up to 60 min). Fitting of such an experimental sequence in the 4-state Kok model yields an S-state population consisting of 36–41% S_0, 40–49% S_1, 1–10% S_2 and up to 13% S_3. No abnormality under the first flash is seen in such preparations. Characteristic for sequences with Oscillatoria preparations is a high level of misses which are in the region of 25 per cent, whereas double hits do not seem to play a substantial role in the damping of such sequences. The existence of metastable S_3, anyway inconsistent with the coherent Kok model, is not confirmed by mass spectrometry. No $^{18}O_2$ seems to be evolved under the first flash from Oscillatoria thylakoids suspended in 50% $H_2{}^{18}O$, although, when judged from the absolute amperometric signal amplitude, mass spectrometric detection of O_2 should have been possible. With the same method we are fully able to detect $^{18}O_2$ under the second flash in *Chlorella vulgaris*. In *Chlorella* this is true for experimental conditions in which the amperometric signal amplitude under the second flash is even smaller than those under the first or second flash in Oscillatoria. The attempt to correlate the amperometrie signal observed under the first flash with a photoinhibition of respiration in our prokaryotic organism was not successful. However, the attempt to incorporate the phenomenon in the coherent Kok model shows that the Oscillatoria sequence fully resembles those with *Chlorella*, if the first flash signal and 40–50% of the signal observed under the second flash is simply removed from the sequence. The remaining sequence exhibits the usual properties known from *Chlorella* or higher plant chloroplasts. If one assumes contribution of the reduced state S_{-1} to the dark population of S-states, a fit in the five rank Kok model yields correct adjustments with a S-state distribution of 6–20% S_{-1}, 31–40% S_0, 49–54% S_1, 0% S_2 and 0% S_3 which would be fully consistent with the Kok model and corresponds to the distribution observed with *Chlorella* or higher plant chloroplasts. The question what the first electrochemical signal is due to remains unanswered.

Introduction

Thibault has repeatedly shown that oxygen evolution measured as the consequence of short saturating flashes in *Chlorella* or higher plant chloroplasts exhibits an abnormality under the first flash [1–3] when the flash sequence is analyzed in the frame of the four state "Kok-Model" [4]. The disturbances of the mathematical analysis of such a flash sequence disappears if the first flash is simply excluded from the analysis [2] or if contribution of a more reduced state than S_0, namely S_{-1}, to the initial dark population of S-states is assumed [3, 5]. This abnormality gains further interest by three recent reports of the literature. Thus, Lavorel and Seibert have observed that in photosystem II particle preparations from spinach the first flash of a sequence yields a positive electrochemical signal which is attributed to a metastable S_3-state [6]. On the other hand Vermeglio and Carrier [7] have shown that in the photosynthetic bacterium *Rhodopseudomonas sphaeroides* the fast-response-electrode-system, as we use it, gives a positive amperometric response under the first flash, although by principle

Abbreviations. SHAM, Salicylhydroxamic acid, CCCP, Carbonylcyanid-*m*-chlorophenyl hydrazone.

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this signal cannot be oxygen, since *Rhodopseudomonas* has no photosystem II. Consequently, the signal was interpreted as being due to a photoinhibition of oxygen uptake in the bacteria which in turn is due to interference of respiration with photosynthesis the carriers of which are located in the same membrane [7]. The third kind of report comes from Bader and Bader and Schmid who have repeatedly mentioned that the oxygen-evolution pattern measured as the consequence of short saturating flashes in the filamentous blue-green alga *Oscillatoria chalybea* is different in comparison to that of *Chlorella* [8–10]. *Oscillatoria* was chosen because filamentous blue green algae are from an evolutionary point of view very different from higher plants or green algae [11] and seem to exhibit as consequence of this fact a whole series of peculiarities in their light metabolism [8, 9]. They are prokaryotes but in contrast to photosynthetic bacteria they are able to produce oxygen because they have photosystem II. The fact that the photosynthetic and respiratory chains are located in the same membrane, just as in photosynthetic bacteria, has among other reasons prompted Stanier to term this class of organisms cyanobacteria [12]. In *Oscillatoria* it is observed that the first flash even after a prolonged dark adaptation results in a measurable and substantial positive amperometric signal which is usually higher than that under the second flash. In comparison to *Chlorella* such a flash sequence shows in addition the difference that maximal flash yield is seen under the fourth flash. Due to the exposed characteristics of blue-green algae the observed oxygen evolution pattern in *Oscillatoria* might contain the elements of the observation by Lavorel and Seibert [6] concerning photosystem II particle preparations, as well as those by Vermeglio and Carrier, namely the interference of photosynthetic and respiratory electron transport during a short light flash [7]. We, therefore, have focussed our effort on the characterization of the electrochemical signal under the first flash, with the aim to prove or disprove that this signal under the first flash is oxygen.

Materials and Methods

Plant material

Oscillatoria chalybea was obtained from the algal collection in Göttingen and cultured in the medium D of Kratz and Myers [13]. The cells were cultured in petri-dishes in which a clay plate was just immerged in the nutrient medium. The *Oscillatoria* cells were growing near the nutrient surface on the clay plate. The cultures were grown in a not climatized room with a 14 h light/10 h dark cycle. Illumination was done with approximately 4500 ergs $\cdot s^{-1} \cdot cm^{-2}$.

Preparation of protoplasts and thylakoids from cells of Oscillatoria chalybea

The harvested cells were incubated in 0.6 M mannitol for 30 min with 0.05% glucuronidase (Boehringer, W.-Germany) at 37 °C in the light. This treatment is supposed to remove the mucoid coating from the cells. Thereafter the filaments were spun down and incubated again in 0.6 M mannitol for 60 min with 0.05% lysozyme (Sigma, USA) and 0.3% cellulase "Onozuka R10" from Kinki Yakoult Manufact. Ltd. Japan. The incubation was carried out at 37 °C in the light. This treatment essentially digests the cell walls of the algae. The obtained suspension was filtered through glass wool in order to separate filament pieces from protoplasts. After three washings with sea salt solution (33 g/l) the protoplasts were spun down (10 min in a cooled clinical centrifuge) and suspended in a buffer containing 0.05 M Tris, 0.01 M NaCl, 0.4 M sucrose, 0.2% bovine serum albumine and 0.2% pectinase pH 7.5. From aliquots of this suspension chlorophyll was determined. Until use in the experiment, the preparation was stored at 0 °C in the dark. Before use the protoplasts were osmotically disrupted by diluting the protoplast suspension approximately 10 times according to Lehmann-Kirk *et al.* [14].

Oxygen measurements

The measurements were carried out by polarography with the three electrode system described by Schmid and Thibault [15]. The electrode system was equipped with a Tektronix 5115 Oscilloscope and with a peak measuring device from Novelec (France). Flashes were provided by a Stroboscope 1539A of General Radio or by the flashing device No. PS 302 from EG and G Inc. (Boston Mass.). The flash duration was at half intensity in the first case 8 μs and 2 μs in the latter case. Usually a sequence of 30 flashes was given, spaced 300 ms apart. The Stroboscope 1539A of General Radio was modified

by changing the only present discharge capacitor of 1 μF by several interchangeable condensators in order to change the light intensity of the flash.

Mass spectrometry

For mass spectrometry *Chlorella* cells or thylakoids of *Oscillatoria chalybea* were suspended in 0.05 M tricine and 0.12 M KCl pH 7. In the presented experiment the suspension volume consisted of approx. 50% of $H_2{}^{18}O$. The total suspension volume did not exceed 3 ml. In another experiment the final pellet of the thylakoid preparation was suspended in 97% $H_2{}^{18}O$. The measurements were carried out in a home made mini-cell directly connected to the ion-source of a Varian Mat CH4 Mass Spectrometer. This mini-cell corresponds, except some modifications, to that used by Dimon [22]. In particular it should be noted that the membrane surface of the cell was 12.6 cm^2.

Results

In comparison to *Chlorella* oxygen evolution in *Oscillatoria chalybea* induced by short (2 or 8 μs) saturating flashes shows some peculiarities. It appears that there always is an appreciable electrochemical signal under the first flash (Figs. 1 a and b), a fact which has already been mentioned earlier by Bader and Bader and Schmid [8, 10]. This signal is usually higher than the one observed under the second flash. Two types of sequences can be observed: In one type the amplitudes of the signal under the first and the second flash are especially high in comparison to those under the third and the fourth flash (Fig. 1 a) whereas the ratio of the amplitudes under the first and second flash in comparison to the maximal flash yields is more normal in the second type of sequences (Fig. 1 b). A further principal observation in *Oscillatoria* sequences is the fact that maximal flash yield always occurs under the fourth flash (Figs. 1 a and b). All these observations are valid for dark adapted preparations (15 min or more). It is obvious that the heart of the problem is the signal under the first flash. Two hypotheses are available for experimental testing. Firstly: The observed signal is no oxygen evolution but rather a photo-induced inhibition of oxygen uptake of the type described by Vermeglio and Carrier for *Rhodo-*

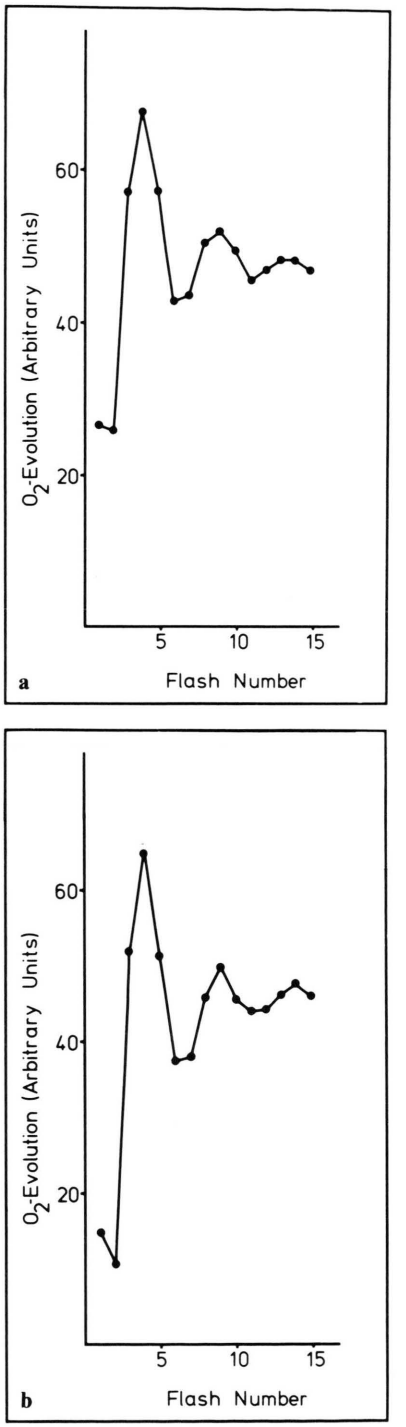


Fig. 1. Two types of flash sequences observed with thylakoid preparations of *Oscillatoria chalybea*. Flashes were spaced 300 ms apart. a) Sequence type with high amplitudes for Y_1 and Y_2, despite the dark adaptation of 20 min. b) Sequence type with lower amplitudes for Y_1 and Y_2; dark adaptation 15 min.

pseudomonas sphaeroides [7]. This possibility is the first to be tested since *Oscillatoria* is an organism which has photosynthetic and respiratory electron transport functioning in the same membrane. The alternative would be that the signal is indeed oxygen. In this case the sequence types of Figure 1a and b would belong to those already observed by Lavorel and Seibert [6] with photosystem II particle preparations from spinach. This latter possibility must be considered for the reason that thylakoid preparations of *Oscillatoria* are in fact a kind of photosystem II particle preparation, since blue-green algae and in particular *Oscillatoria* have in comparison to higher plants a remarkable excess of photosystem II over photosystem I activity as shown by fluorescence studies [9, 11]. Besides this excess of photosystem II activity over that of photosystem I, also a certain degree of disconnection between the two photosystems is general [16]. If the signal under the first flash in photosystem II particle preparations or in Oscillatoria is oxygen, one obvious implication of the observation would be the existence of metastable S_3-state which in context with the existing coherent model of oxygen evolution [4] would be of no merit.

I. Experimental attempt to prove that the first signal is due to an inhibition of respiration in Oscillatoria chalybea

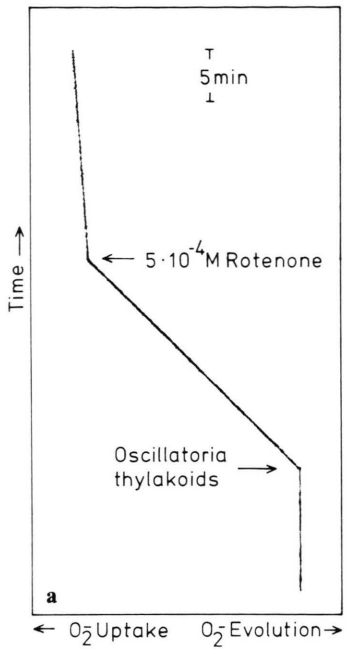
Under the assumption that the first amperometric signal in the *Oscillatoria* flash sequence is no net oxygen evolution, the influence of various respiration inhibitors upon the flash pattern was tested. The reagents used were Rotenone, CCCP and KCN. When studying respiration in blue-green algae the principal fact that dark respiration is always very low in these organisms, must be taken into account [16]. This circumstance was verified for *Oscillatoria* (Table I). It is seen that dark respiration in *Oscillatoria* or *Anacystis* is under no condition higher than that of *Chlorella* under comparable growth conditions. Dark respiration in *Oscillatoria* is nearly fully inhibited by Rotenone (Fig. 2a) which according to the literature is supposed to block respiratory electron transport in the region of complex I [17]. Under the condition where this inhibition is effective the flash sequence pattern of *Oscillatoria* remains unchanged *i.e.* the signal under the first flash is still present (Fig. 2b). Vermeglio and Carrier have

Table I. Dark respiration in various photosynthetic organisms.

	Respiration
	[μmol O_2 uptake · mg chlorophyll^{-1} · h^{-1}]
Chlorella vulgaris	15 ± 7
Oscillatoria chalybea	6 ± 2
Anacystis nidulans	10 ± 4
Rhodospeudomonas sphaeroides	150 ± 24[a]

[a] per Bacteriochlorophyll; temperature 25 °C. Variations are absolute maximal variations induced by differing conditions such as measurements before and after a light period.

reported that CCCP (10 μM) in *Rhodopseudomonas sph.* causes a stimulation of oxygen uptake *i.e.* a relief of inhibition under the second or every even flash which was due to the uncoupling properties of the agent [7]. Using this agent we expected, accordingly, a modification in the signal pattern of the first two flashes or in detail a faster relaxation kinetic under the second flash. This was not the case. Fig. 3 shows the effect of two CCCP-concentrations on the flash sequence. The amplitudes of all signals are affected with 10 μM CCCP; doubling this concentration supresses the signals under all flashes fully. An effect of suitable lower CCCP-concentrations on the relaxation kinetics of the first two flashes and in particular on the second flash is seen under no condition. The effect of CCCP as we observe it, is an effect on the amplitudes of the signal which follows in *Oscillatoria* exactly the same concentration dependency as that reported by Thibault for *Chlorella* [3]. Fig. 4 shows the effect of 5 μM CCCP on the relaxation kinetics of the first 4 flashes. It is clearly seen that the amplitudes of all four signals are affected in comparison to the control. It seems as if the effect on the amplitude in the third and fourth flash is accompanied by a reduction of the fast kinetic region of the biphasic signal. No acceleration of the relaxation kinetic of the second flash is observed (Fig. 4). It seems obvious that the effect of KCN or Sham would bring the decisive information provided these agents affect respiration in our organism. It appears that Sham has no effect whatsoever on our system whereas KCN in concentrations starting from 10^{-5} M already affects photosystem II, *i.e.* the amplitudes of steady state oxygen evolution in our



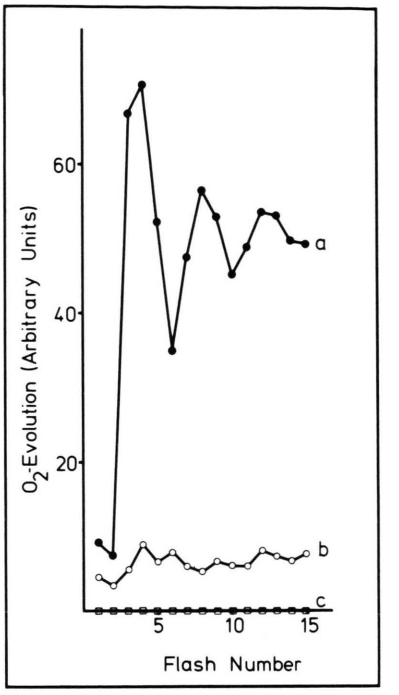


Fig. 3. Effect of 10 μM CCCP (○) and 20 μM CCCP (□) on the flash sequence of *Oscillatoria chalybea*. (●) control sequence. Dark adaptation 15 min.

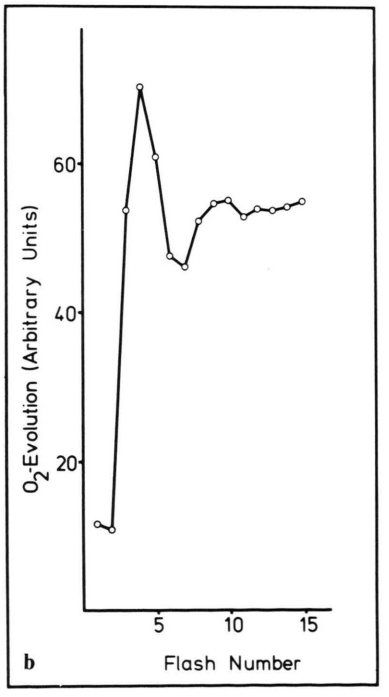


Fig. 2. a) Effect of 5×10^{-4} M Rotenone on dark respiration in *Oscillatoria chalybea*; b) Flash sequence in the presence of 5×10^{-4} M Rotenone with *Oscillatoria chalybea* thylakoids dark adapted for 20 min.

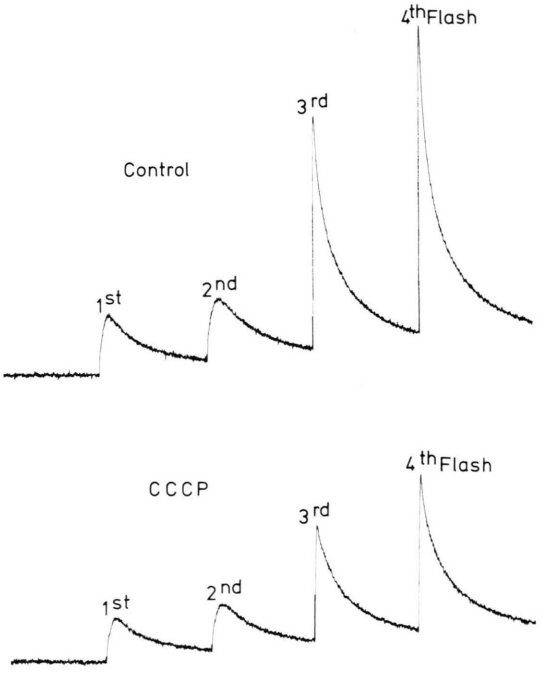


Fig. 4. Effect of 5×10^{-6} M CCCP on the relaxation kinetics of the first 4 electrochemical signals in *Oscillatoria chalybea*. Note effect on the amplitudes as well as on the relaxation kinetics in comparison to the control.

sequence pattern. KCN concentrations which usually affect dark respirations ($\sim 10^{-3}$ M [7]) induce in our organism in the light a slow but very high oxygen uptake which makes an analysis of the anyway reduced signals senseless. This type of light induced oxygen uptake in the presence of KCN has already been described in the literature [18]. Another obvious way to demonstrate a relationship of the first two flash signals with respiration seemed to us the removal of oxygen from the thylakoid suspension of the algae. Oxygen cannot be taken up when not present. Three approaches have been made in this direction: Flushing with nitrogen; addition of β-D-Glucose-oxidase to the suspension, an oxygen scavenger known from the literature [19], and addition of a suspension of the bacterium *Rhodopseudomonas sphaeroides* to the thylakoid suspension of our organism.

Flushing of the suspension with N_2 would lead to a special sensitivity of the first two signals, should the respiration hypothesis apply. The effect of nitrogen on the flash sequence pattern seems twofold. A short flushing of up to 10 min does not change the principal features of the control sequence although an effect on the amplitudes caused by annoxia is quickly seen. But all amplitudes are affected in proportion with no special consequence detectable in the first two signals. No effect on the relaxation kinetics of the first four signals is seen when compared to the control. The proportion of the fast and slower phase stays within a given signal the same. Extensive flushing with nitrogen (25 min or more) however, changes the sequence pattern in an abrupt manner (Fig. 5). The first two signals are suppressed and maximal flash yield is retarded by one flash into the fifth flash which seems consistent with the reduction of the oxygen-evolving system as a whole into a more reduced condition [3].

Addition of β-D-glucose-oxidase to the thylakoid suspension let us expect a special effect on the first two signals whereas the rapid O_2-evolution of the third and further flashes seemed to us too rapid to be effectively scavenged by the enzyme. To our surprise high enzyme concentrations were able to suppress all signals completely (Fig. 6). Lower concentrations suppressed all signals to the same extent with no preference for the first and second one. Hence, the experiment did not permit the discrimination between the first and second signal on the one side and the third and fourth on the other.

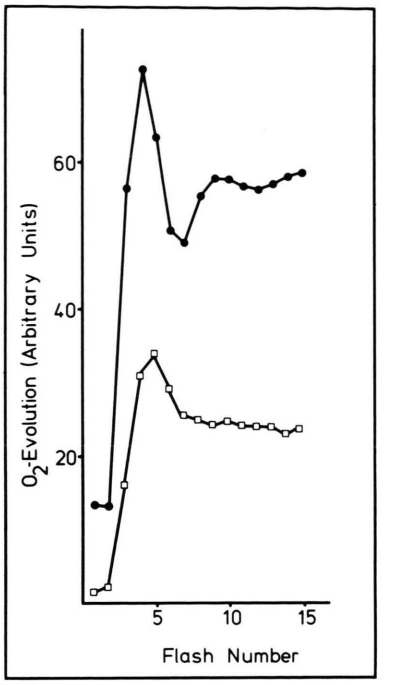


Fig. 5. Effect of flushing with nitrogen on the oxygen evolution pattern of flash sequences in *Oscillatoria chalybea*. (□) 25 min N_2-flushing; (●) control in air.

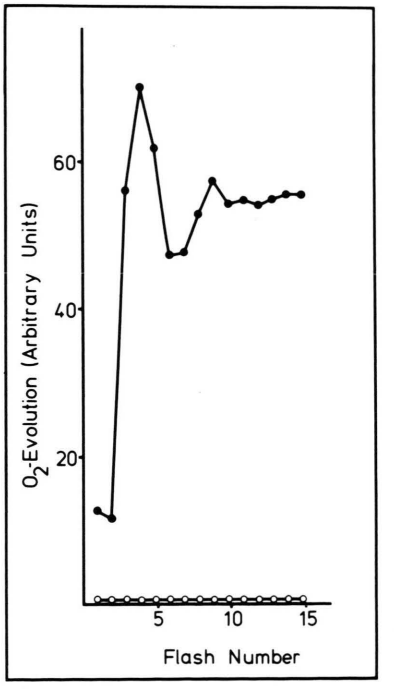


Fig. 6. Effect of β-D-glucose-oxidase (Sigma) on the oxygen-evolution pattern of flash sequences in *Oscillatoria* (●) control; (○) in the presence of 100 units/assay enzyme and 10^{-2} M glucose.

Addition of Rhodopseudomonas sphaeroides to the thylakoid suspension gave essentially the same results as the experiment with Glucose oxidase. The original aim was to study the Vermeglio and Carrier [7] type of photoinduced inhibition of oxygen uptake in the presence of high and low quantities of the thylakoid preparation of *Oscillatoria chalybea*. We observed that *Rhodopseudomonas sphaeroides* has a dark respiration rate higher by one to two orders of magnitude than *Oscillatoria chalybea*. The bacterium is so eager to take up oxygen that addition of suitable amounts of bacteria cells to the thylakoid preparation of *Oscillatoria chalybea* is capable of suppressing all oxygen signals fully. Lower concentrations of bacteria diminish the oxygen signals of the sequence to the same extent with no preference of the first two signals just as with β-D-glucose-oxidase. However, this type of experiment permitted us to realize that the size of the photoinduced inhibition of oxygen uptake in *Rhodopseudomonas sphaeroides* which we can fully confirm, produces a positive amperometric signal 5 to 20 times smaller than the one discussed in this paper for *Oscillatoria chalybea*. In conclusion, the presented experiments did not permit us to demonstrate that the first electrochemical signal of our flash sequence in *Oscillatoria chalybea* is not oxygen. It seems to us, especially taking into acaount the low respiratory activity of our organism, that respiration cannot be the ultimate cause of the electrochemical signal observed under the first flash.

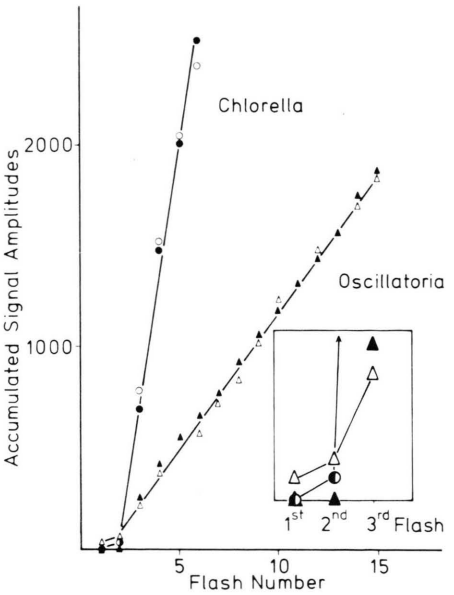


Fig. 7. Oxygen evolution measured as electrochemical signal and by mass spectrometry of mass 36 in *Chlorella vulgaris* and *Oscillatoria chalybea*. *Chlorella*: (○) electrode signal; (●) mass spectrometry. *Oscillatoria* (△) electrode signal; (▲) mass spectrometry. The inset refers to the first three flashes.

II. Experimental attempt to prove that the first flash signal in Oscillatoria chalybea is due to oxygen evolution

1. Mass spectrometry: The cleanest way to prove that the first electrochemical signal in the flash sequences of *Oscillatoria chalybea* (Fig. 1) is indeed oxygen, would be to show appearance of masses attributable to one of the oxygen isotopes $^{16}O_2$ or $^{18}O_2$ after exposure to one single flash. We have marked the thylakoid suspension of *Oscillatoria chalybea* to approximately 50% with $H_2{}^{18}O$ and have measured $^{18}O_2$-evolution in a usual flash sequence of 15 flashes. We have compared the obtained evolution pattern with the electrochemically obtained sequence. For comparison purpose we have run the same experiment also with *Chlorella*. The observations are summarized in Fig. 7. It is seen that $^{18}O_2$-evolution follows within the understandable error width the electrochemical sequence fully as far as *Chlorella* is concerned. Detection of $^{18}O_2$ under the second flash was no major problem. In *Oscillatoria*, however, we did not observe $^{18}O_2$ under the first flash and at most only traces under the second flash although the electrochemical sequence gave already under the first flash a signal as high as that under the second flash in *Chlorella*. Since the method determines the accumulated evolutions at a given flash number, the observed signal under the second flash should have been consistently higher than under the second one in *Chlorella*. However, $^{18}O_2$-evolution under the second flash was in *Oscillatoria chalybea* within detection limits equally zero. Hence, mass spectrometry supports the idea that the first amperometric signal of the flash sequence in *Oscillatoria chalybea* is no oxygen evolution or at least not oxygen evolved due to photosynthetic water splitting.

2. Polarogram: Determination of the dependence of the first sequence signal upon different polarization voltages shows very similar behaviour in com-

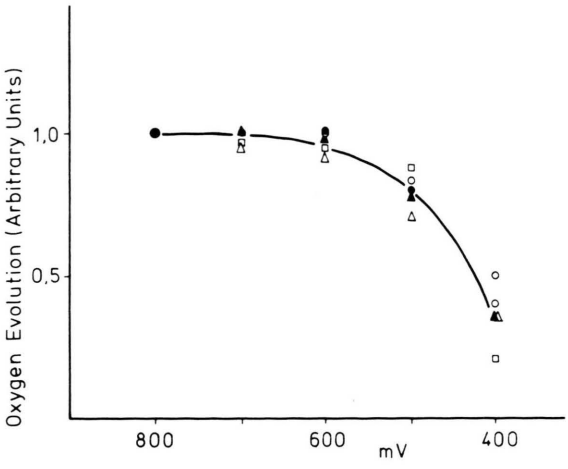


Fig. 8. Polarogram; Dependence of the first four flash signals in *Oscillatoria chalybea* on the polarization voltage. (○) first flash signal; (●) second flash signal; (△) third flash signal; (▲) fourth flash signal; (□) For comparison purpose the signal under the third flash in *Chlorella vulgaris* is shown. All values are initially normalized to the value at 800 mV.

parison to the same dependence of an authentic oxygen signal and does not permit the conclusion that the first signal is not oxygen (Fig. 8). Due to the mass spectrometry experiment the question arises where this oxygen would come from.

3. Comparison of the first four electrochemical signals in Oscillatoria chalybea with those in Chlorella

If one compares the shapes of the first amperometric signals in *Oscillatoria chalybea* with those of *Chlorella* no principal or major difference is detectable at first glance (Fig. 9a and b). Especially the signal properties of the first two flashes in *Oscillatoria chalybea* seem fully comparable to the signal under the second flash in *Chlorella* which nobody would question to be oxygen. The kinetic analysis of the signals gives for both organisms the choice of two first order kinetics per signal or of one second order reaction per flash (Fig. 9c). Hence, if the signal under the second flash in *Chlorella* is due to oxygen, the first two *Oscillatoria* signals according to the type of experiment should be oxygen too, a result which would support the polarogram (Fig. 8).

Sensitivity of the flash sequence pattern to hydroxylamine

If *Oscillatoria* thykaloids are treated with hydroxylamine the sequence pattern is changed as shown in Fig. 10. Maximal flash yield is observed under the seventh flash, with the first four to five flashes giving a low but constant positive signal. The fact that the first four signals are not zero seems to us artefactual and due to the presence of the cyanobacterial mucoids which retard effective penetration of the chemical. Addition of cyanobacterial mucoid preparations to *Chlorella* cells has similar effects under these conditions. The effect of hydroxylamine is consistent with the reduction of the positive charge accumulation complex to state S_{-2} [3]. Disregarding a one quantum requirement for the separation of a reduced reaction-centre-hydroxylamine complex [20] S_{-2} requires the absorption of 6 quanta to evolve oxygen [3]. One remarkable feature of *Oscillatoria* sequences is their sensitivity also to low concentrations of hydroxylamine. Already 1 micromolar or lower concentrations of hydroxylamine have in dark adapted preparations an effect on the S-state distribution although such a flash sequence has visually barely undergone any change or effect. Only the mathematical analysis reveals that especially the S_3-population of the control has been diminished. In this context it should be noted that hydroxylamine has no effect on dark respiration in *Oscillatoria chalybea*. Summarizing, the properties of the flash sequence towards hydroxylamine could be used to speak in favor of oxygen evolution under the first flash and would support the interpretation of Lavorel and Seibert [6].

Incorporation of the phenomenon into a coherent model of photosynthetic oxygen evolution

The classical reference model for oxygen evolution is the Kok model [4]; four successive charge accumulations corresponding to the successive transition of state S_0 to S_1, S_2 and S_3 lead to oxygen evolution. State S_0 is dark stable, S_1 is practically dark stable whereas S_2 and S_3 fully relax in the dark. Practically all reasonable observations on photosynthetic oxygen evolution can be interpreted in the sense of this model. In the following we attempt to incorporate the phenomenon *i.e.* the sequence pattern of Fig. 1 into this model. Clearly, two trivial possibilities exist: the first signal is due to oxygen evolution or it is not.

1. Assumption, the observed first signal is oxygen. If one starts out with the possibility that the signal

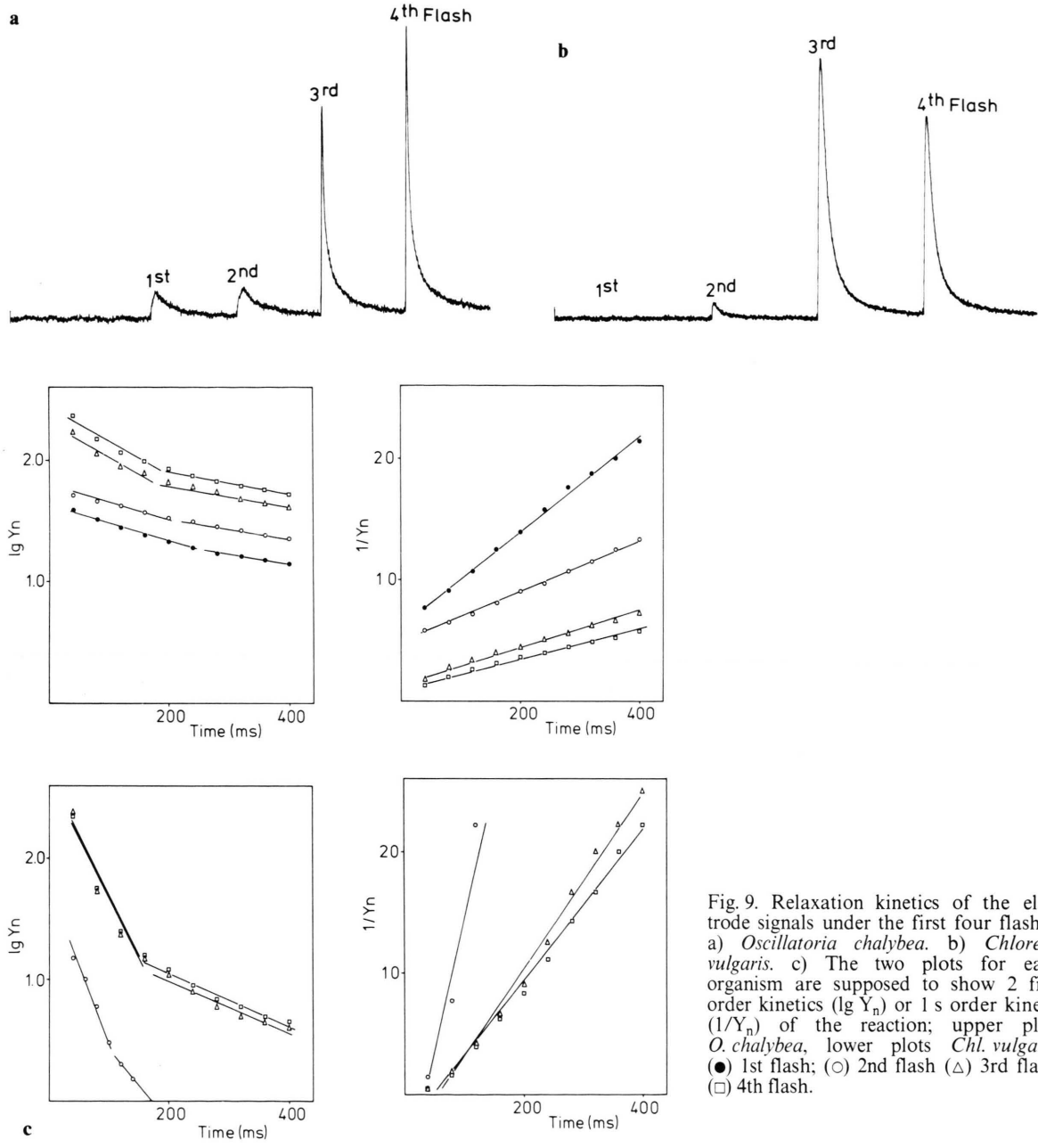


Fig. 9. Relaxation kinetics of the electrode signals under the first four flashes. a) *Oscillatoria chalybea.* b) *Chlorella vulgaris.* c) The two plots for each organism are supposed to show 2 first order kinetics ($\lg Y_n$) or 1 s order kinetic ($1/Y_n$) of the reaction; upper plots *O. chalybea*, lower plots *Chl. vulgaris.* (●) 1st flash; (○) 2nd flash (△) 3rd flash; (□) 4th flash.

under the first flash is oxygen, the implications of the observation are the following: Since the sequences of Fig. 1 a and b are obtained with dark adapted material a positive signal under the first flash means metastable S_3, an observation and interpretation already made by Lavorel and Seibert for spinach system II particle preparations [6]. Calculation of the S-state distribution for sequences 1 a and b in the sense of the 4 state Kok model according to the principles described by Lavorel [21], Thibault [3] and Thibault and Thiery [5] yields the result shown in Table II. The S-state population is above all characterized by 7–12 per cent S_3 which in the sense of the coherent model is not easily acceptable.

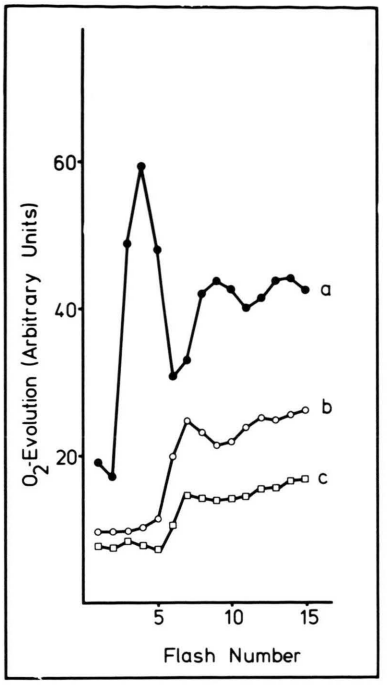


Fig. 10. Flash sequence of *Oscillatoria chalybea* in the presence of 50 μmolar (○) and 100 μmolar (□) hydroxylamine. (●) control sequence, dark adapted for 15 min.

In detail, however, it should be noted that the fit in the 4-state Kok model is correct (Fig. 11 a and b). In fact, the fit of the experimental sequences of Fig. 1 a and b by means of the four state Kok model is much better than those obtainable for *Chlorella* which according to Thibault [3] is due to the possibility that contribution of a more reduced state than S_0, namely S_{-1}, to the initial dark population might exist in *Chlorella* and higher plant chloroplasts. Consequently, a *Chlorella* sequence gives much better results when the experimental sequence is fitted by means of a "five state" Kok model [3]. Thibault observed that approximately 17% of the relaxed dark S-state population is made up of S_{-1} [3]. Fitting of the sequences of Fig. 1 a and b in the five state Kok model (Table III) does not modify the result shown in Table II and above all does not improve the quality of the fit (Fig. 11 c and d). It thus appears that a sequence of the type shown in Fig. 1 represents much purer Kok 4 conditions than *Chlorella* with practically no abnormality under the first flash at all. Despite this fact, which is adjustability of the sequence in the 4-state Kok model, we feel that metastable S_3 is no acceptable interpretation. No valid experimental case is known that

Table II. S-State population in thylakoid preparations of *Oscillatoria chalybea* calculated from a fit in the 4-state Kok model.

Sequence	S_0	S_1	S_2	S_3	Misses α	Double hits γ	Δ%
				(in per cent)			
1a	36.2	40.2	10.9	12.7	25.3	1.8	1.16
1b	41.6	49.9	1.0	7.5	26.5	3.5	0.6

Dark adapted for 15 min. Sequence 1a and 1b are identical with those shown in Fig. 1. Δ% relative quadratic deviation.

Table III. S-State population in thylakoid preparations of *Oscillatoria chalybea* calculated from a fit in the 5-state Kok model.

Sequence	S_{-1}	S_0	S_1	S_2	S_3	Misses α	Double hits γ	Δ%
					(in per cent)			
1a	2.9	34.2	38.8	11.7	12.4	24.6	0.57	1.4
1b	−1.3	42.2	50.9	0.4	7.6	25.3	1.8	0.7

Same sequences and conditions as in Table II.

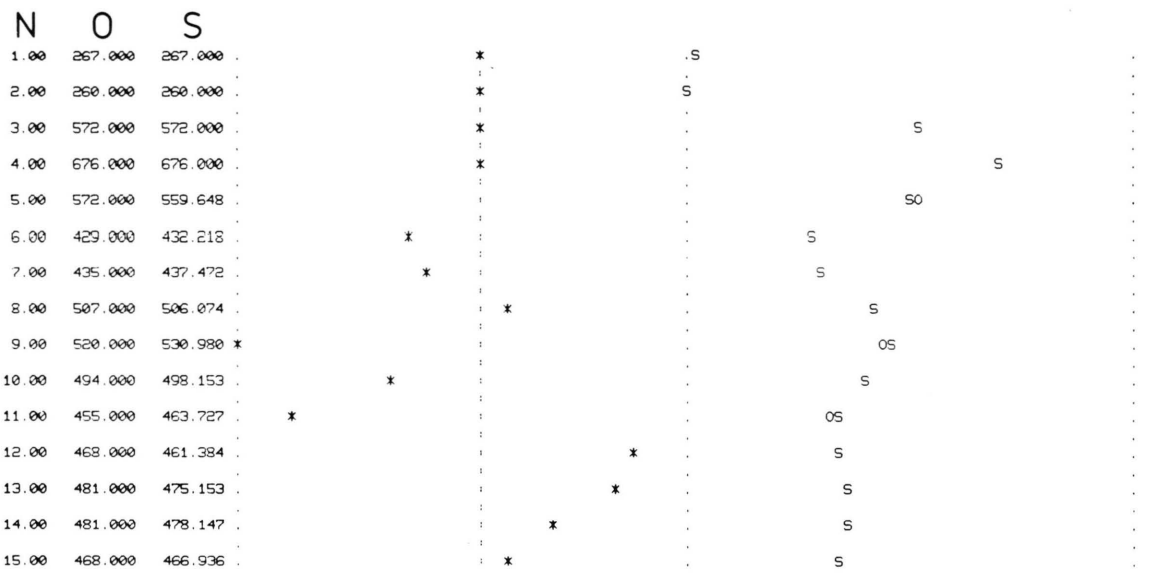


Fig. 11a

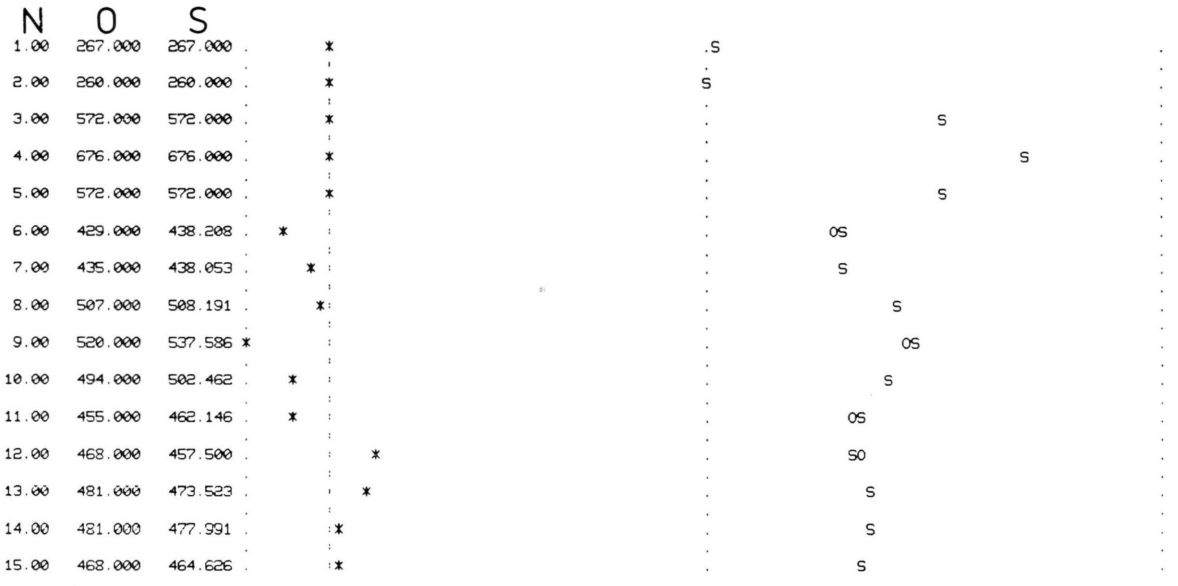


Fig. 11b

would have shown that S_2 was more unstable than S_3.

2. Evaluation of the possibility that the first flash signal is not due to oxygen evolution. Under the assumption that Y_1 is not oxygen, one can as the first possibility simply exclude this signal from the mathematical fitting of the sequence in the *four state Kok model.* The procedure yields a theoretical and reasonable solution which however exhibits some problems concerning the stability of S_2 (Table IV). The next obvious step is to look for interference with respiration. If a phenomenon of the type reported by Vermeglio and Carrier [7] applies, an oscillatory phenomenon with a periodicity of two would be contained in a sequence like

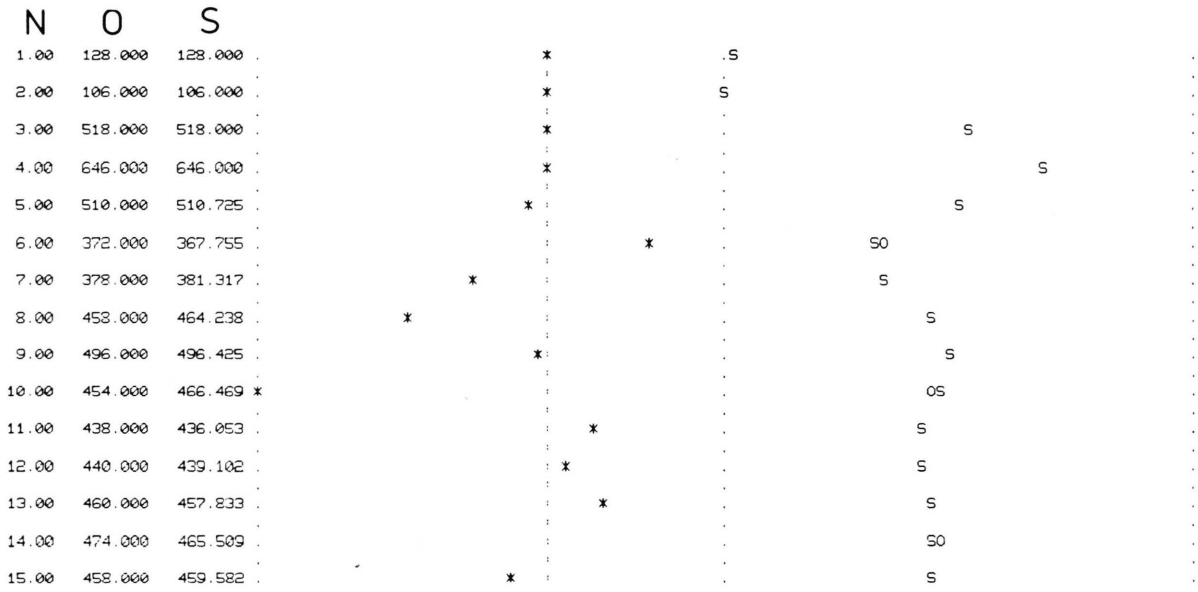


Fig. 11c

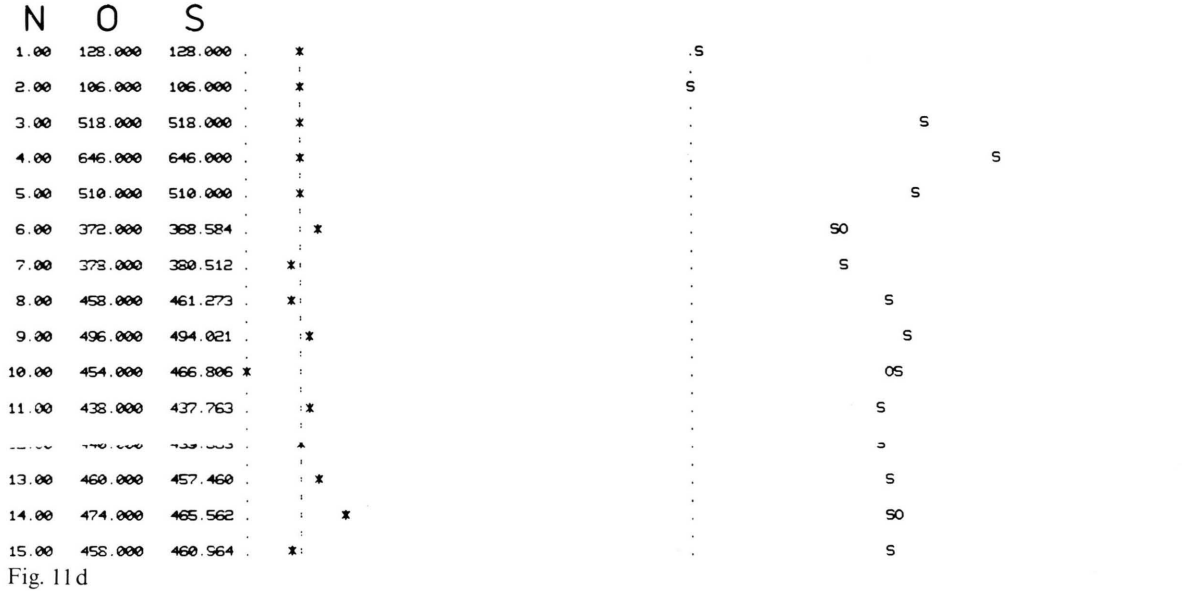


Fig. 11d

Fig. 11. Fit of experimental sequences by means of the Kok model; the right hand picture shows the quality of the fit. (S) are simulated points, (O) are observed points. Where (S) and (O) coincide only S is printed. The left hand pictures show the deviations which are multiplied with a factor as indicated: a) Sequence adjusted for Fig. 1a in the four state Kok model. The deviations are multiplied by 30; b) Sequence adjustment for Fig. 1a in the five state Kok model. Note that 4 simulated points (S) do not coincide with the observed (O) ones which means that the quality of the fit is not better than in a) Deviations are multiplied by 6; c) Sequence adjustment for Fig. 1b in the four state Kok model. The deviations are multiplied by 40; d) Sequence adjustment for Fig. 1b in the five state Kok model. Deviations are multiplied by 8. **N** denotes the column for the flash numbers, **O** denotes the respective observed flash amplitudes and **S** the simulated ones.

that of Fig. 1. We have, therefore, subtracted Y_1 from all uneven flash signals and Y_2 from the even ones and then fitted the sequence in the four state Kok model. This really leads to no reasonable result and strictly worsens everything. The next approach was to say, not only $Y_1 = 0$ but also a fraction of Y_2 is not due to oxygen. If in addition to Y_1 one gradually takes off 1/4, 1/2, 3/4, 4/4 of Y_2 and fits thereafter the sequence in the four state Kok model the result shown in Table IV is gradually worsened, yielding solutions that become more and more inacceptable. But if one scrutinizes carefully these results obtained with sequences in which Y_1 and part of Y_2 has been excluded from the treated sequence one realizes that the S-state population is very similar to those obtained with *Chlorella* or chloroplasts in which the sequences have been adjusted with "Kok 4" [3]. These sequences exhibit as Thibault has demonstrated an abnormality under the first flash [1, 2]. This abnormality is thought to be either due to an increased rate of double hitting under the first flash [1] or rather due to the contributon of the more reduced state S_{-1} to the initial dark population [2, 3]. Adjustment of the *Chlorella* sequence in a five state Kok model makes the abnormality fully disappear. Double hitting plays no role for the discussed phenomenon in *Oscillatoria* since essentially the same sequence is observed when the flash source is a 10 ns laser. Consequently, we have fitted the sequences of Fig. 1 a and b after subtractions of Y_1 and increasing portions of Y_2 in this five state Kok model (Table V).

Table IV. S-State population in thylakoid preparations of *Oscillatoria chalybea* calculated from a fit in the 4-state Kok model after the removal of Y_1 and fractions of Y_2 from the sequence.

Sequence	Condition	S_0	S_1	S_2	S_3	Misses α	Double hits γ	Δ%
			(in per cent)					
1 a	$Y_1 = 0$; 1/1 Y_2	44.4	41.9	14.7	−1	29	4.6	2.6
1 b		45.2	52.4	2.6	−0.2	29	4.4	0.9
1 a	$Y_1 = 0$; 3/4 Y_2	45.4	43.4	6.8	−0.7	30	6.2	2.2
1 b		45.5	55.5	−1.1	0.08	29	5	1.06
1 a	$Y_1 = 0$; 1/2 Y_2	45.7	57.5	−3.7	0.5	31	7.9	2.46
1 b		45.7	59.3	−5.4	0.5	29	5.6	1.23
1 a	$Y_1 = 0$; 1/4 Y_2	44.9	70.8	−18.9	3.2	31	10	2.85
1 b		45.7	63.5	−10.2	1.0	30	6.3	1.4
1 a	$Y_1 = 0$; $Y_2 = 0$	42.3	91.3	−43.4	9.8	33	12.2	3.3
1 b		45.6	68.2	−15.5	1.7	30	7	1.6

Δ% Relative quadratic deviation.

Table V. S-State population in thylakoid preparations of *Oscillatoria chalybea* calculated from a fit in the 5-state Kok model after the removal of Y_1 and fractions of Y_2 from the sequence.

Sequence	Condition	S_{-1}	S_0	S_1	S_2	S_3	Misses α	Double hits γ	Δ%
				(in per cent)					
1 a	$Y_1 = 0$; 1/1 Y_2	14.2	32.2	36.6	16.7	0.3	25	−1.3	2.1
1 b		4.5	41.2	50	4.4	−0.2	27	3.1	0.7
1 a	$Y_1 = 0$; 3/4 Y_2	15.4	32.7	40.0	11.9	−0.04	25	2	1.6
1 b		5.5	4.1	52.1	1.5	−0.07	27	3.4	0.7
1 a	$Y_1 = 0$; 1/2 Y_2	17.0	32.6	43.9	6.6	−0.14	25	1.6	1.2
1 b		6.6	40.6	54.4	−1.7	0.09	27	3.9	0.7
1 a	$Y_1 = 0$; 1/4 Y_2	19.2	31.9	48.2	0.7	−0.02	25	2.7	1.09
1 b		7.8	40.0	56.8	−4.9	0.3	27	4.2	0.7
1 a	$Y_1 = 0$; $Y_2 = 0$	21.9	30.4	53	−5.7	0.3	25	3.7	0.9
1 b		9.05	39.3	53.3	−8.2	0.6	27	4.6	0.76

Δ% Relative quadratic deviation.

The result is surprising in the sense that the quality of the fit is very good, above all in comparison to the results in Table IV. In the case of sequence 1a an optimal solution is recognizable in the region between 3/4 and 1/2 Y_2, whereas in the case of sequence 1b the solution is probably that in which 1/2 Y_2 had been subtracted. These two acceptable solutions for the sequences 1a and b can be deduced from Table V. They represent solutions which would be fully coherent with the hypothesis of the dark lability of the states S_2 and S_3. Moreover, they are practically identical for both types of sequences and are fully comparable to results usually obtained with *Chlorella* [3]. Thus, it seems as if Y_1 was no oxygen and only a fraction of the Y_2 signal would be oxygen. Thus, in the case of sequence 1a 60% of the electrochemical signal under the second flash and in sequence 1b 50% would be due to oxygen evolution. Interestingly, in both cases the size of the real Y_2 corresponds to 11% of the flash signal under steady state conditions (0.11 Y_{ss}) just as in *Chlorella*.

Discussion

If a usual flash sequence of *Chlorella* or chloroplasts is fitted with the four state Kok model, a substantial abnormality is observed under the first flash [1–3]. This abnormality is easily overcome if Y_1 is simply left out. If one asks the question how a flash sequence, well adjustable in the four state Kok model, should look without this abnormality, the answer is Y_1 should be positive and have approximately 60% of the steady state signal (Y_{ss}). In a first attempt Thibault has interpreted the actual situation in *Chlorella* (no or only a very small signal under the first flash) as being due to 50 to 60 per cent of double hitting under the first flash [1]. In a second approach Thibault realized that the abnormality also disappeared if (in *Chlorella* for example) contribution of a more reduced state than S_0, namely S_{-1} to the initial dark state population was assumed. The state corresponds to a condition of the reaction center in which five light quanta are needed to evolve oxygen. In *Chlorella* 17% of the initial dark S-state population is S_{-1}. Hence, the abnormality disappears if chloroplast or *Chlorella* sequences are adjusted in a five rank instead of a four rank model [3, 5]. In the present paper we presented the paradox that in the filamentous blue green alga *Oscillatoria chalybea* the flash pattern, as observed, is adjustable in the four rank model. The implication of this result, however, would be the existence of metastable S_3 since we can experimentally exclude the double hitting hypothesis (the flash sequence does not change if the flash source is a 10 ns laser). As we disliked the idea of metastable S_3 we thought first of interference of respiration and photosynthesis [7] in our organism which is a prokaryote. Although we are practically certain that interference of respiratory with photosynthetic electron transport is not at the origin of our signal under the first flash, we observe that the sequence becomes adjustable in the five rank model after removal of Y_1 and $0.4-0.5\ Y_2$ from the sequence. The results then look very much like *Chlorella*. This paradox between adjustability in the four rank and five rank model is further stressed by the comparison of the experiments by mass spectrometry which convincingly show that Y_1 is no oxygen and those of the polarogram type which do not disprove that Y_1 is oxygen. The question thus arises what Y_1 might be due to. If the first signal comes from oxygen it is certainly not due to oxygen evolved by photosynthetic water splitting from $H_2{}^{18}O$, but should come from bound oxygen that did not exchange with $H_2{}^{18}O$. If the first signal is not caused by oxygen at all it must be due to a redox component of the membrane, capable of extracting electrons from the platinum electrode the half wave potential of which would be very similar to that of oxygen. Caused by the preparation procedure, photosystem II particle preparations as those described by Lavorel and Seibert [6] or our thylakoid preparations of *Oscillatoria* [9] might represent special membrane conditions in which such a redox component becomes active.

Acknowledgements

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