Studies on the S-State Distribution in *Euglena gracilis*

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When *Euglena gracilis* is dark adapted for 10 min or more, oxygen evolution as the consequence of short (5 μsec) saturating light flashes does not show the picture of a damped oscillation with a periodicity of 4, as known from the literature. The overall picture of this flash pattern is given by the fact that O_2-evolution in the first two flashes is practically zero and rises from there onward in a continuous manner to the steady state with barely any visible oscillation at all. However, a second flash sequence fired one to two minutes after this first sequence induces an oxygen evolution pattern which is barely distinguishable from the well known usual *Chlorella vulgaris* pattern. The phenomenon is not influenced by changes in the oxygen tension nor do additions of chemicals like CCCP, sodium azide, or reducing agents like hydroxylamine or hydrogen peroxide substantially alter the described behavior. Deactivation experiments give the overall impression that the deactivation of the S-states is slower than with *Chlorella*. Hydroxylamine strongly accelerates the deactivation. The analysis of the S-state distribution in a four and five state Kok-model suggests that dark adapted *Euglena* is in a more reduced condition than dark adapted *Chlorella*. It looks as if dark adapted *Euglena* were in a condition which would correspond to 60 percent S_{-1}, 30 percent S_0 and 10 percent S_1. The experimental flash sequence of such dark adapted cells fits best a synthetic sequence when the misses are in the region of 20–25 percent, with double hitting playing practically no role at all (the first two flashes are zero!). The impression that dark adapted *Euglena* starts its oxygen evolution from a more reduced state is strengthened by the analysis of room temperature fluorescence induction (Kautsky effect). It can be shown that the fluorescence induction curve of *Euglena* corresponds to that of *Chlorella* cells provided the latter have been briefly treated with a strong reductant such as sodium dithionite.

Introduction

Photosynthetic O_2-evolution measured as the consequence of short saturating light flashes shows the picture of a damped oscillation with the periodicity of four [1, 2]. The molecular interpretation of this observation has produced a great series of models [1–5]. From all these models the so called Kok model describes in a relatively simple way the accumulation of the four positive charges in each trapping center of photosystem II (S-state model) and offers the best possibilities to compare model predicitions with a whole series of experimental data. Thus, Thibault realized that the comparison of experimental data with the Kok-model yielded a substantial abnormality under the first flash [6]. In a first attempt this was interpreted by an increased rate of double hits under the first flash [6]. Further studies concerning this abnormality have been interpreted rather in the sense of the contribution of a more reduced state S_{-1} to the usual initial S-state population [7]. It should be noted, that the existence of such a state has been felt already by Kok and coworkers from effects of reducing agents such as hydrogen peroxide on flash sequences [8]. The present paper gives some evidence that in dark adapted *Euglena gracilis* the S_{-1}-state or an equivalent condition might prevaile in comparison to S_0 and S_1.

Abbreviations: DCMU, N-N′-3,4 dichlorophenyldimethylurea; SHAM, salicylhydroxamic acid; CCCP, carbonylcyanid-*m*-chlorophenyl hydrazone.

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Materials and Methods

Euglena gracilis was cultured at 30 °C in glass tubes bubbled with 2% CO_2 in air and illuminated by 9000 lux white light. The culture medium was that by Cramer and Myers [9] supplemented with Vitamin B_{12} 50 μg/l and Vitamin B_1 100 μg/l as well as with trace elements: $MnCl_2 \times 4\ H_2O$, 1.8 mg/l; $ZnSO_4 \times 7\ H_2O$, 400 μg/l; $CuSO_4 \times 5\ H_2O$, 20 μg/l; $Na_2MoO_4 \times 2\ H_2O$, 200 μg/l; $CaCl_2 \times 6 H_2O$, 1.2 mg/l and $Co(NO_3)_2 \times 6\ H_2O$, 1.3 mg/l. For the assay

washed cells were suspended in 0.02 M phosphate buffer pH 7.2.

Oxygen measurements: The measurements of oxygen evolution were carried out by polarography with the three electrode system described by Schmid and Thibault [10]. 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 1539 A 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 μsec and 2 μsec in the latter case. Usually a sequence of 30 flashes was given spaced as indicated either 300 or 600 msec apart. The Stroboscope 1539 A 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.

Fluorescence induction was measured with a self designed device which was assembled by Secia, Manosque, France. The measuring system corresponds essentially to that described by Joliot *et al.* [11]. The exciting light was filtered through a blue Schott (Mainz) BG 28 filter. Fluorescence emission was measured in the reflection mode, selected by a monochromator and the 691 nm emission detected by a photomultiplier PM: EMI 9558 QB. The fluorescence device was equipped with a Tektronix 5115 memory oscillscope.

Results

a. O_2-Evolution

If one measures oxygen evolution in suitable dark adapted *Chlorella vulgaris* (*e.g.* 20 min), one observes the result shown in Fig. 1. This pattern is manifold described in the literature [12] and contains the following facts: there is practically no O_2-evolution in the first flash, some in the second, with the maximum O_2-evolution observed in the third flash. Moreover, one observes the picture of a damped oscillation with the periodicity of four [1, 2]. If one analyses this pattern by means of the four state Kok model in the usual way which is a fitting of the experimental data by variation of the S-state population and by variation of the transition probabilities in the model, the result equally known from the literature is obtained: in dark

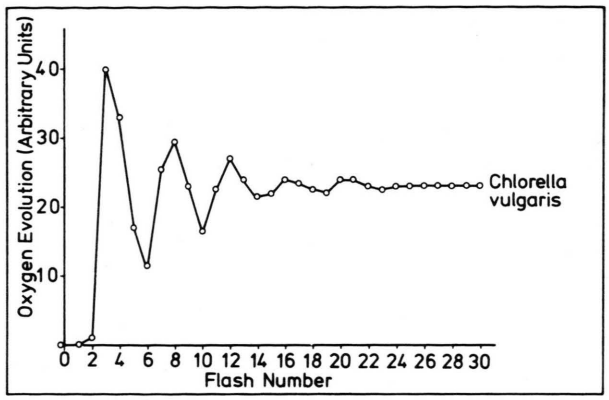

Fig. 1. *Chlorella vulgaris* suspension dark adapted for 20 min before firing a flash sequence of 30 flashes spaced 300 msec apart.

adapted Chlorella the oxygen-evolving system is found to be in a condition which corresponds to 75% S_1 and 25% S_0. The damping of the oscillation corresponds to approximately 79 percent successful transitions (transition probability β); approximately 15 percent of the reaction centers remained, despite the flash, in the same state (transition probability α) and were missed by the flash (misses) whereas approximately 6 percent of the centers were excited twice during the life time of the flash thus advancing by two steps (states) towards oxygen evolution (double hit, transition probability γ). The sum of these three transition probabilities stays according to the Kok model 1.

If the same experiment is carried out with a strain of *Euglena gracilis* which we believe to correspond to the wild type, we observe the flash pattern shown in Fig. 2a. After 20 min of dark adaption no periodic oscillation of the type shown in Fig. 1. is seen. Oxygen evolution is almost zero in the first two flashes. Firing of a second flash series two to four minutes after this first sequence yields a normal flash pattern which at the first glance is practically not distinguishable from the usual *Chlorella* pattern (Fig. 2b). At first we thought that wrong experimental conditions such as lack of oxygen or CO_2 and others were the reason of the present observation but we were able to exclude these possibilities completely. According to the literature anoxia diminishes the transition probability α [7]. However, lowering or rising the oxygen tension does not alter the phenomenon. According to the literature addition of sodium azide [14] improves the quality of

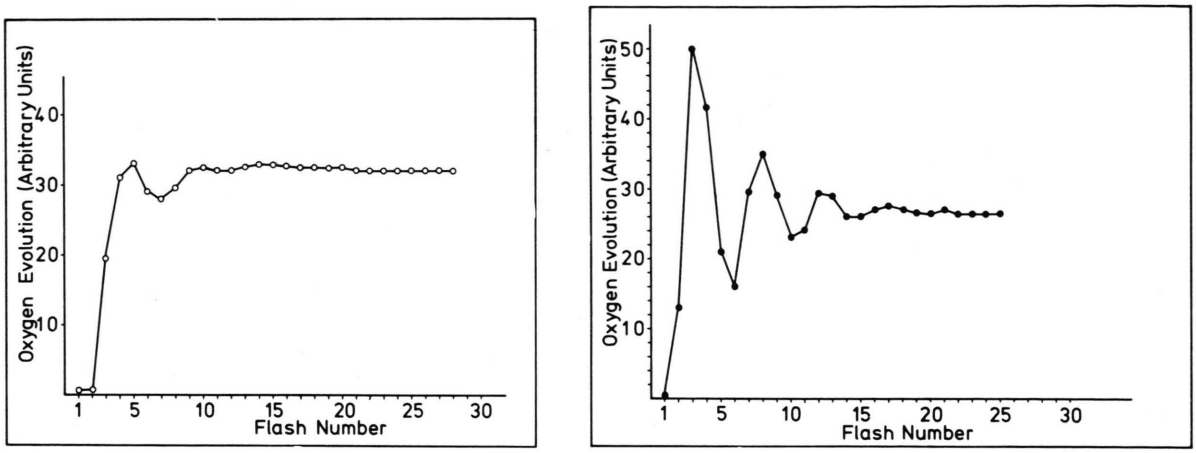

Fig. 2. a) Suspension of *Euglena gracilis* dark adapted for 20 min before firing a flash sequence under identical conditions as in Fig. 1. b) Flash sequence obtained with the same suspension of *Euglena gracilis* as that used in Fig. 2 a. The flashes were fired 1 min after the series which yielded Fig. 2 a.

the oscillation which was not the case for the described experiment. CCCP blocks charge recombinations of the reaction center [15] and causes oscillations to be more sustained [13]. The overall impression of Figs. 2 a and b was not changed by adding CCCP to the cell suspension. We looked for effects with a series of chemicals which one would obviously test in such a case namely KCN, SHAM, antimycine, *p*-benzoquinone, NADPH, H_2O_2 etc. These chemicals had on the phenomenon itself (Fig. 2 a and b) no effect. Their final effect on O_2-evolution as soon as the control sequence showed the *Chlorella* pattern, was that described in the literature for *Chlorella*.

Treatment of dark adapted *Chlorella* with 50×10^{-6} M hydroxylamine leads according to Bouges-Bocquet [16] to a shift of the usual O_2-sequence by three flashes. One observes in this case onset of the usual *Chlorella* sequence after 3 flashes which yield no oxygen-evolution at all.

The same experiment carried out with *Euglena gracilis* in the presence of 50 or 70 µM NH_2OH leads at the first glance again to no appreciable effect on the above described phenomenon (Fig. 3). One observes two flashes with no O_2-evolution and then a gradually increasing O_2-evolution until the steady state is reached. Under the condition that hydroxylamine is not metabolized by *Euglena* in an unusual manner, the explanation for this observation can didactically only be that dark adapted *Euglena* is from the beginning in a more reduced state than *Chlorella*. It looks as if the condition of dark adapted *Euglena* corresponds somehow to the *Chlorella* condition in the presence of hydroxyl amine. A close-up scrutiny of Fig. 3 and comparison to Bouges-Bocquet's hydroxylamine effect [16] leads after all to the observation that in dark adapted *Euglena* in the presence of hydroxylamine although

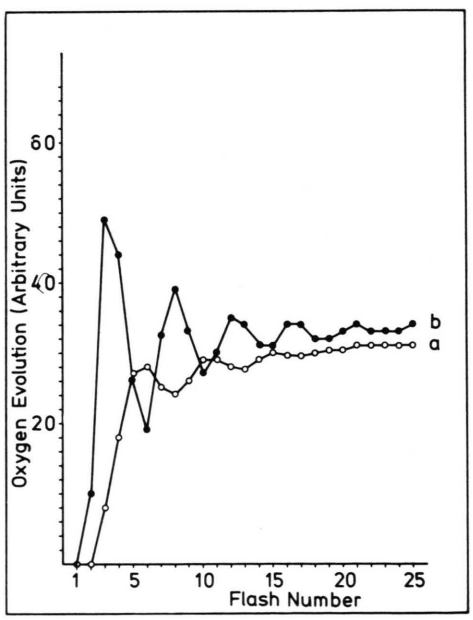

Fig. 3. Flash sequence of *Euglena gracilis* in the presence of 50 µmol hydroxylamine. a) Dark adapted for 12 min. b) Flash sequence fired 1 min after 3 a.

the first two flashes yield no oxygen as in the control, the maximal flash yield seems to be retarded by one flash in the presence of hydroxylamine (Figs. 2a and 3a). Aside from this modest observation our interpretation is at this point not more than an unproven hypothesis, above all since our attempt to transfer dark adapted *Euglena* with chemical oxidation reagents (for example $KMnO_4$) into the condition of dark adapted *Chlorella*, was not successful.

In order to better characterize the *Euglena* system we have studied the deactivation of the S-states. Fig. 4 shows the deactivation of the S-states as well as the effect of 50 µM hydroxylamine on the deactivation by plotting the respective oxygen amplitudes (y_i) against the dark times between the flash sequences. The principal effect of hydroxylamine is the clear acceleration of the deactivation of all states.

With the four oxygen amplitudes $Y_{1,2,3,4}$ of Fig. 4 at a given time we have made a mathematical fit on the "shape factors", by means of the recurrence law established by Lavorel [17].

In the present context the authors should like to note, that the classical fitting method described for Fig. 1 is overdetermined *i.e.* redundant. There, the amplitudes of an entire sequence (*e.g.* 15–30 flashes) together with the transition probabilities are used. However, according to Lavorel [17] or Thibault and Thierry [18] a sequence can be fully synthesized with the O_2-amplitudes of the first four

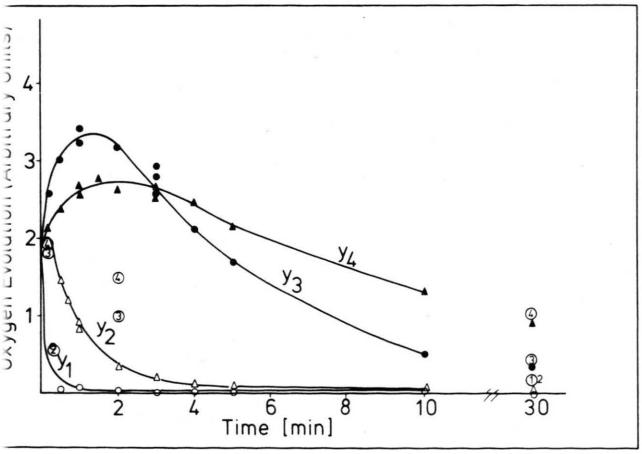

Fig. 4. Yield of oxygen in the first four flashes y_1 to y_4 of a sequence in dependence on the dark time between the flash sequences in *Euglena gracilis*. The flashes were spaced 300 msec apart. The encircled numbers are oxygen yields for the respective flash numbers in the presence of 50 µmol hydroxylamine.

or five flashes by making the fit on these shape factors. Since $\alpha + \beta + \gamma = 1$, the liberty degree is 2. This method permits the extension of the Kok-model to state numbers greater than 4, without changing the number of independent parameters. Practically this extension is limited to 6 states.

① $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3$

② $S_{-1} \rightarrow S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3$

③ $S_{-2} \rightarrow S_{-1} \rightarrow S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3$

Scheme of the linear Kok-model ① and its extension by the introduction of state S_{-1} ② and of state S_{-2} ③.

Since Thibault [7] and Thibault and Thiery [18] have found out that the curve fitting with *Chlorella* yields better results with ② in the sense that the model with rank 5 (type ② of the scheme) describes the experimental series with a lower least square error deviation and above all without an error oscillating systematically with the flash number [7, 18] we have calculated the S-state deactivation for the ② type of the Kok model. The result is shown in Fig. 5 which shows the deactivations of the S-states in dependence on the dark time between the flash sequences. From steady state, deactivation of the S-state system is supposed to start with a condition equivalent to $S_0 = S_1 = S_2 = S_3 = 25\%$ and $S_{-1} = 0\%$.

Fig. 5 shows clearly that after more than 2 min of dark adaptation the state S_{-1} gains importance, whereas S_1 becomes smaller and smaller. At the 10 min marker of the graph S_{-1} is present in the concentration equivalent to that of S_1 after 40 sec. It is interesting to note that in agreement with the visual estimate of Fig. 2a the misses α increase with time whereas the double hits γ do not increase to the same extent. The latter statement is to a certain degree already obvious from Fig. 2a where it is seen that oxygen evolution in the first two flashes is zero. The deactivation with *Euglena* cells shows in comparison to the general properties of *Chlorella* a slow time course (factor 2–3). Otherwise, at the first glance, no major pecularity is seen in comparison to *Chlorella*. However, a comparison with the thesis of Thibault [7], which refers only to *Chlorella* cells, shows that with *Chlorella* in long

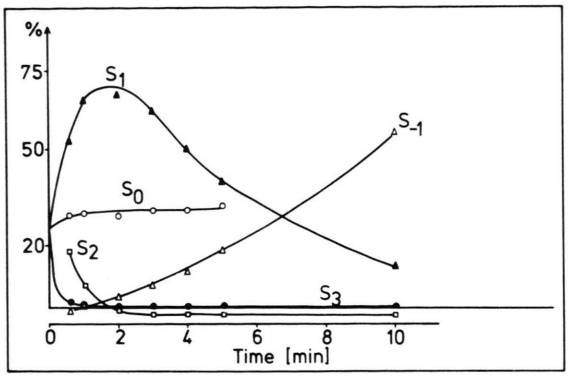

Fig. 5. Deactivation of S-states in *Euglena gracilis.*

term experiments not many changes are observed. After 10 min for example S_2 and S_3 just as in Fig. 5 are not anymore existant. S_{-1} is in this case after 60 min as well as already after 10 min in the region of 17%; S_0 between 20 and 30% with S_1 after 10 or 60 min* unchanged at around 60%. In this respect our Fig. 5 yields the following balance: After 10 min S_{-1} has apparently at the expense of S_1 increased up to the order to 50 percent, S_0 remains constant as with Thibault [7] at approximately 30%. Moreover Table I shows the deactivation values or the S-state distribution of a typical experiment with *Euglena gracilis* after 12 min of darkness. The table clearly shows the inversion of the states S_1 and S_{-1} in comparison to *Chlorella* [7] which we can fully confirm for dark times up to 100 min. Thus, Fig. 5 together with Table I might show that in *Euglena* dark relaxation leads to a more reduced condition or state which would be S_{-1} at the expense of S_1. From the effect of the reducing agent H_2O_2 on the O_2-evolution of chloroplasts the existence of such a state was suspected by Kok and coworkers [8] many years ago. Since sufficiently dark adapted *Euglena* is apparently from the beginning in a comparable condition it becomes almost evident that reducing agents such as H_2O_2 or NH_2OH cause no additional effect on the distribution of the S-states or at least do not change a flash sequence pattern in the fashion described by Bouges-Bocquet [16].

* Long term values in *Euglena* are characterized by a lack of "shape" (high values for α). The values in Table I, however, are not to be considered as being not precise enough since we obtain such values starting from flash sequences with more "shape" at shorter times in a continuous manner.

b. The Kautsky effect in Euglena gracilis

If one studies room temperature fluorescence induction of dark adapted *Chlorella*, one observes at medium time resolution of the induction (measurement over 1 sec, Fig. 6a) the biphasic rise kinetics with the subsequent fluorescence decrease well known from the literature [19]. Measurement of the induction over 50 sec yields a steep rise of the fluorescence with a subsequent decrease to the steady state (Fig. 6a, 5 s/sq). The study of *Euglena* fluorescence under the same conditions yields the fluorescence course shown in Fig. 6b. At first glance the induction with *Euglena* appears much faster because of the fact that the *Chlorella* curve taken with a sweep speed of 5 s/sq looks very similar to that with *Euglena* taken at a speed of 100 ms/sq. The fluorescence shown in Fig. 6b is influenced by DCMU in the manner shown in Fig. 6c. In the course of further studies we were able to show that after an extended dark adaptation the fluorescence observed with the same sample is much lower than with shorter dark adaptations (Fig. 6d). This is again an observation which has been manifold described for *Chlorella* and which at first appears trivial. However, the extent of the effect (Fig. 6d) appears noteworthy to us. In the attempt to simulate a *Euglena* type fluorescence induction (Fig. 6b) with chemical means in *Chlorella*, one observes, that a fluorescence induction shown as that in Fig. 6b is

Table I. Comparison of S-state population in *Euglena gracilis* and *Chlorella vulgaris* after 12 min of darkness.

	S_1	S_2	S_3	S_0	S_{-1}	Misses [%]	Double hits [%]
			[in percent]				
Euglena gracilis	12.46	− 3.85	0.82	30.21	60.35	30	9.1
Chlorella vulgaris	52	− 0.4	0	31.2	17.2	16	6

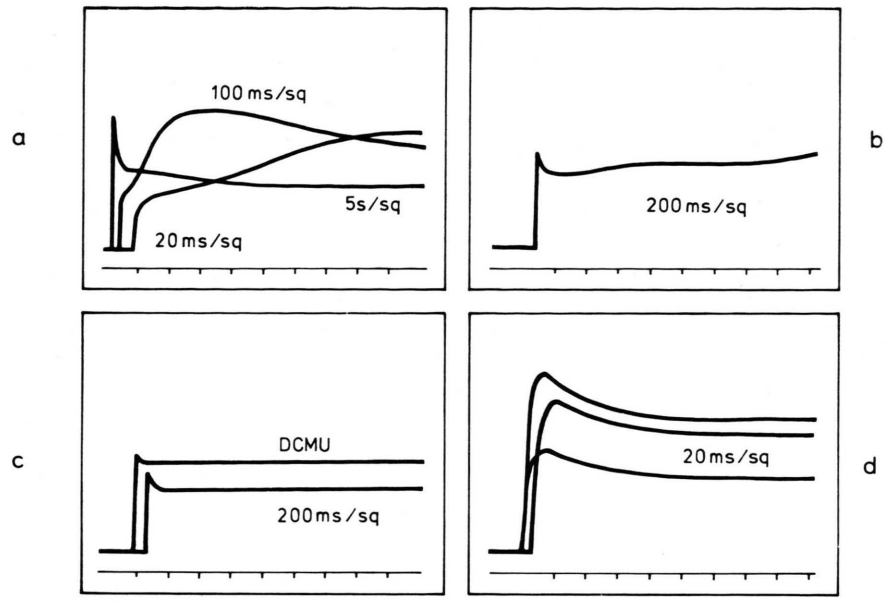

Fig. 6. Fluorescence induction kinetics in *Chlorella vulgaris* and *Euglena gracilis* suspensions of identical chlorophyll concentrations. a) *Chlorella vulgaris:* Appearance of the Kautsky effect of the same suspension in dependence of the time resolution of the induction kinetics. The sweep speed is indicated in seconds per division (s/sq). Dark adaptation 10 min. b) *Euglena gracilis.* Kautsky effect with a time resolution of 200 msec per division. c) Effect of DCMU 2×10^{-6} molar on the fluorescence induction in *Euglena gracilis.* d) Effect of different dark adaptations on the fluorescence yield in *Euglena gracilis.* Lower curve 12 min, middle curve 7 min and upper curve 1 min.

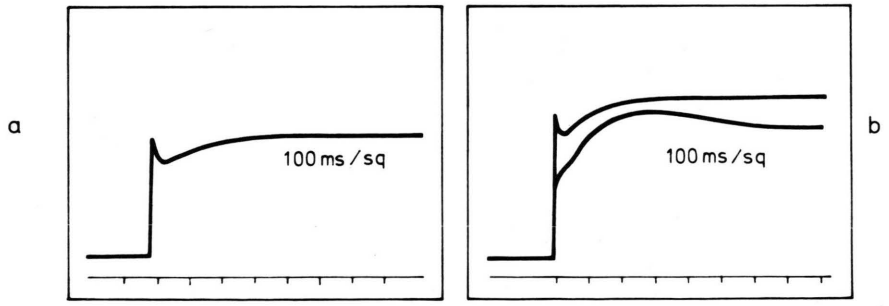

Fig. 7. a) Kautsky effect of 12 min dark adapted *Euglena gracilis.* Time resolution 100 msec per division. b) Kautsky effect of 12 min dark adapted *Chlorella vulgaris;* lower curve without additions, upper curve suspension which yielded the lower curve after the addition of a few crystals of sodium dithionite ($Na_2S_2O_4$). Time resolution 100 msec per division.

induced in *Chlorella* if this alga has been briefly treated with the strong reductant sodium dithionite (Fig. 7a).

Under these conditions the *Chlorella* fluorescence induction corresponds to that in the native state with dark adapted *Euglena* (Fig. 6b and 7b). This is taken as further evidence for the contention, that after a suitable dark adaptation, photosystem II in *Euglena* is in a more reduced condition than in *Chlorella* or at least in a state which can be also be obtained with *Chlorella* with the strong reductant $Na_2S_2O_4$.

Conclusion

In the present paper we were able to show that in dark adapted *Euglena gracilis* the oxygen-evolving

system finds itself in a more reduced condition than in dark adapted *Chlorella*. We have attributed this property to the prevailance of the more reduced state S_{-1} in dark adapted *Euglena*. It appears as if in *Euglena* S_{-1} reaches at the expense of S_1 levels of up to 60% whereas well adapted *Chlorella* reaches a final level of at best 20% S_1 (7). How this special redox environment is obtained is not yet clear, but it seems as if the ultrastructural delimination of the *Euglena* chloroplast towards the cytoplasm is different from that of *Chlorella* (Ruppel personal communication). As to the properties of this new state S_{-1} it should be noted that in dark adapted *Euglena* 60% of the reaction centres must subsequently absorb 5 quanta in order to evolve molecular oxygen and would find themselves thereafter in the state S_0. S_{-1} is supposed to be a new redox state of the positive charge accumulation complex which in *Euglena* by dark relaxation derives itself from S_1 but needs exogenous reducing agents in *Chlorella* in order to be accumulated in appreciable amounts.

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